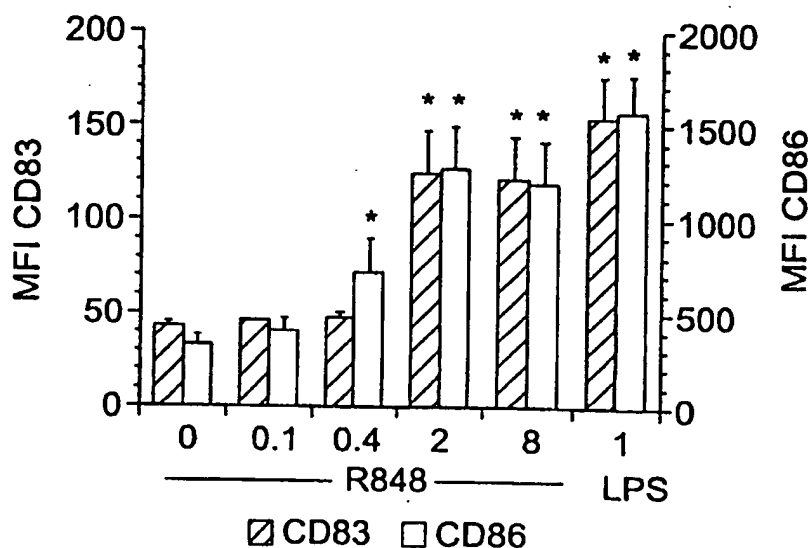




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 5/00		A2	(11) International Publication Number: WO 00/47719
			(43) International Publication Date: 17 August 2000 (17.08.00)
(21) International Application Number: PCT/US00/00757 (22) International Filing Date: 12 January 2000 (12.01.00) (30) Priority Data: 09/248,439 11 February 1999 (11.02.99) US (71) Applicant: 3M INNOVATIVE PROPERTIES COMPANY [US/US]; 3M Center, P.O. Box 33427, Saint Paul, MN 55133-3427 (US). (72) Inventors: TOMAI, Mark, A. ; P.O. Box 33427, Saint Paul, MN 55133-3427 (US). VASILAKOS, John, P. ; P.O. Box 33427, Saint Paul, MN 55133-3427 (US). AHONEN, Cory, L. ; P.O. Box 33427, Saint Paul, MN 55133-3427 (US). (74) Agents: HOWARD, MarySusan et al. ; 3M Innovative Proper- ties Company, Office of Intellectual Property Counsel, P.O. Box 33427, Saint Paul, MN 55133-3427 (US).		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (Utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published Without international search report and to be republished upon receipt of that report.	

(54) Title: MATURATION OF DENDRITIC CELLS WITH IMMUNE RESPONSE MODIFYING COMPOUNDS



(57) Abstract

A method of inducing the maturation of dendritic cells by stimulating immature dendritic cells with an imidazoquinoline type immune response modifying compound. Dendritic cells that have been matured in this manner display increased antigen presenting ability and may be used as immunotherapeutic agents.

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MATURATION OF DENDRITIC CELLS WITH IMMUNE RESPONSE
MODIFYING COMPOUNDS

Field of the Invention

5 The invention relates to the use of synthetic immune response modifiers to induce the maturation of dendritic cells *in vitro*. The invention additionally relates to methods of maturing dendritic cells, to methods of enhancing the antigen presenting ability of dendritic cells, and of enhancing T-cell stimulation using synthetic immune response modifiers. The invention further relates to cellular adjuvants prepared with the dendritic
10 cells that have been matured according to the method of the invention.

Background of the Invention

 Dendritic cells are known to play an important role in the immune system, both for their potent antigen presenting ability and their ability to initiate T-cell mediated immune
15 responses. Indeed, dendritic cells ("DC") activate T-cells more efficiently than any other known antigen presenting cell, and may be required for the initial activation of naïve T-cells *in vitro* and *in vivo*. These cells are generally present in the body at locations that are routinely exposed to foreign antigens, such as the skin, lung, gut, blood, and lymphoid tissues. In general, DC are broadly classified as immature or mature. Immature DC
20 endocytose and process antigen efficiently, but express low levels of costimulatory molecules. In contrast, mature DC display increased levels of costimulatory molecules CD40, CD80 and CD86, as well as HLA-DR. In addition, mature DC express CD83 and secrete increased amounts of various cytokines and chemokines that aid T-cell activation.

 In addition to naïve T-cell activation, DC can influence the balance of the Th1/Th2
25 immune response. Several reports have indicated that DC preferentially activate Th1 responses, with the major determining factor being IL-12 secretion from the activated DC. Macatonia et al., *J. Immunol.* 154:5071 (1995). Hilkens et al., *Blood* 90:1920 (1997). Other reports have shown that DC can induce the generation of either Th1 or Th2 clones. Roth, et al., *Scand. J. Immunol.* 43:646 (1996). The evidence indicates that multiple
30 factors influence the ability of DC to initiate a Th1 or Th2 response, including the DC to T-cell ratio, the DC tissue of origin, the amount of antigen used to prime the DC, the

expression of costimulatory molecules and the antigen injection route.

The pivotal role played by DC in antigen presentation and T-cell activation has resulted in considerable interest in the use of DC in immunotherapy. This is particularly evident in the areas of vaccinology and cancer immunotherapy. Although much effort has
5 been devoted to the development of successful vaccines using recombinant DNA, successful clinical use of DNA vaccines has not been achieved. Recent evidence indicates that effective immunization with DNA vaccines requires recombinant protein expression from DC. Further, enhanced immunity in animal models has been achieved utilizing DNA
10 vaccines that encode for cytokines or that contain CpG oligonucleotide sequences that upregulate DC maturation. Recently, autologous DC obtained from cancer patients have been used for cancer immunotherapy. See, e.g., WO98/23728. Accordingly, efficient *ex vivo* methods for generating DC are prerequisite for successful immunotherapy.

In general, the process of *ex vivo* DC generation consists of obtaining DC precursor cells and then differentiating the cells *in vitro* into DC before introduction back into the
15 patient. However, the DC must be terminally differentiated, or they will de-differentiate into monocytes/macrophages and lose much of their immunopotentiating ability. *Ex vivo* DC maturation has been successfully accomplished with monocyte conditioned medium; recombinant cytokines such as TNF- α , IL-1 and IL-6; bacterial products such as LPS, bacterial DNA and cross-linking CD40; and transfection with genes that encode cytokines
20 or costimulatory molecules. While these methods are capable of producing mature DC, there are disadvantages to using recombinant molecules and cellular supernatants for maturing DC. These include inconsistent quality and yield from lot to lot of these reagents and the introduction of exogenous proteins into patients, which may be toxic or result in autoimmunity. Such reagents can also be expensive to produce, making the cost of
25 immunotherapy prohibitively expensive. There is a need for a method of maturing DC *in vitro* that is reliable and efficient, without the drawbacks of the currently known methods.

Summary of the Invention

We have found that certain immune response modifier (IRM) compounds can
30 induce the maturation of DC *in vitro*. These compounds are small molecules that can be readily produced at a consistent, high level of purity and potency. By using these

compounds one can efficiently and consistently mature DC, which can then be used as immunotherapeutic agents. The IRM compounds useful in the method of the invention are generally of the imidazoquinoline type; that is, they have a structure that contains the imidazoquinoline ring system or a similar ring system, such as imidazopyridine or
5 imidazonaphthyridine.

Accordingly, the invention provides a method of *in vitro* maturation of dendritic cells comprising treating said dendritic cells with an imidazoquinoline type immune response modifying compound, as well as a population of dendritic cells produced by this method.

10 The invention further provides a method of enhancing the antigen presenting ability of dendritic cells comprising treating said dendritic cells with an imidazoquinoline type immune response modifying compound.

In addition, the invention provides a method of preparing a cellular adjuvant for the treatment of a disease comprising the steps of maturing dendritic cells *in vitro* by treating
15 the dendritic cells with an imidazoquinoline type immune response modifying compound and exposing the mature dendritic cells to an antigen associated with said disease.

Brief Description of the Drawings

Fig. 1 is a graphical depiction of the ability of the IRM compound 4-amino-2-ethoxymethyl- α , α -dimethyl-1H-imidazo [4,5-c] quinolin-1-ethanol (R-848) to enhance
20 cell surface expression of CD83 and CD86.

Fig. 2 shows the ability of R-848 to enhance the cell surface expression of co-stimulatory molecules on MO-DC.

Fig. 3 shows the maturation of DC as measured by cell surface expression of
25 various markers after 6 hours of stimulation with 2 μ g/ml R-848.

Fig. 4 depicts the results of treating MO-DC with R-848 on T-cell proliferation and T-cell cytokine production as seen by a primary MLR.

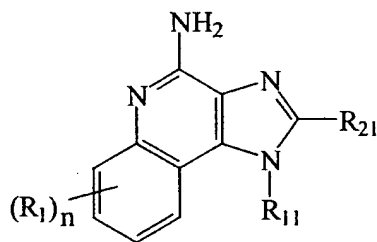
Fig. 5 shows the response of R-848 treated MO-DC to tetanus toxoid.

Detailed Description of the Invention

The IRM Compounds

Compounds useful in the methods of the invention include imidazoquinoline type IRM compounds. In general, the term "imidazoquinoline type IRM compounds" refers to compounds containing an imidazoquinoline ring system or a similar ring system that have the ability to modify the immune response. Preferred imidazoquinoline type IRM compounds contain one or more of the following ring systems: imidazoquinoline; imidazopyridine; 6,7 fused cycloalkylimidazopyridine; 1,2-bridged imidazoquinoline; imidazonaphthyridine; and imidazotetrahydronaphthyridine. Particularly preferred IRM compounds contain an imidazoquinoline-4-amine ring system. Compounds useful in the methods of the invention will also typically have the ability to induce production of one or more of the cytokines TNF- α , IL-1, IL-6 and IL-12 when administered to a host or applied *in vitro* to dendritic cells or monocyte/macrophages.

Immune response modifier compounds useful in the method of the invention include compounds defined by Formulas I-IX(b) below. Preferred 1H-imidazo [4,5-c] quinolin-4-amines are defined by Formulas I-V:



I

wherein

R₁₁ is selected from the group consisting of alkyl of one to ten carbon atoms, hydroxyalkyl of one to six carbon atoms, acyloxyalkyl wherein the acyloxy moiety is alkanoyloxy of two to four carbon atoms or benzyloxy, and the alkyl moiety contains one to six carbon atoms, benzyl, (phenyl)ethyl and phenyl, said benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties

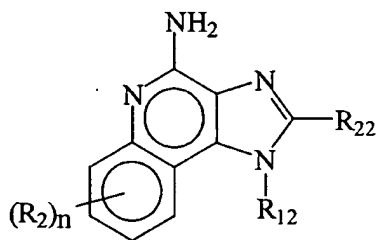
independently selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms and halogen, with the proviso that if said benzene ring is substituted by two of said moieties, then said moieties together contain no more than six carbon atoms;

5 R_{21} is selected from the group consisting of hydrogen, alkyl of one to eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms and halogen, with the proviso that when the benzene ring is substituted by two of said moieties, then the moieties together contain no more than six carbon atoms;

10 and

each R_1 is independently selected from the group consisting of alkoxy of one to four carbon atoms, halogen, and alkyl of one to four carbon atoms, and n is an integer from 0 to 2, with the proviso that if n is 2, then said R_1 groups together contain no more than six carbon atoms;

15



II

wherein

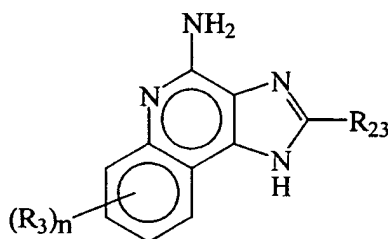
R_{12} is selected from the group consisting of straight chain or branched chain alkenyl containing two to ten carbon atoms and substituted straight chain or branched chain alkenyl containing two to ten carbon atoms, wherein the substituent is selected from the group consisting of straight chain or branched chain alkyl containing one to four carbon atoms and cycloalkyl containing three to six carbon atoms; and cycloalkyl containing three to six carbon atoms substituted by straight chain or branched chain alkyl containing one to four carbon atoms; and

20

25

R_{22} is selected from the group consisting of hydrogen, straight chain or branched chain alkyl containing one to eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of straight chain or branched chain alkyl containing one to four carbon atoms, straight chain or branched chain alkoxy containing one to four carbon atoms, and halogen, with the proviso that when the benzene ring is substituted by two such moieties, then the moieties together contain no more than six carbon atoms; and

each R_2 is independently selected from the group consisting of straight chain or branched chain alkoxy containing one to four carbon atoms, halogen, and straight chain or branched chain alkyl containing one to four carbon atoms, and n is an integer from zero to 2, with the proviso that if n is 2, then said R_2 groups together contain no more than six carbon atoms;



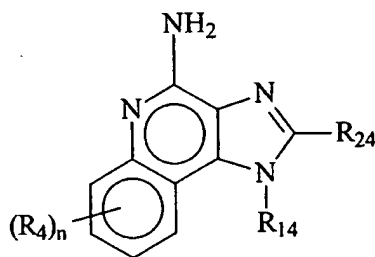
III

wherein

R_{23} is selected from the group consisting of hydrogen, straight chain or branched chain alkyl of one to eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of straight chain or branched chain alkyl of one to four carbon atoms, straight chain or branched chain alkoxy of one to four carbon atoms, and halogen, with the proviso that when the benzene ring is substituted by two such moieties, then the moieties together contain no more than six carbon atoms; and

each R_3 is independently selected from the group consisting of straight chain or branched chain alkoxy of one to four carbon atoms, halogen, and straight chain or

branched chain alkyl of one to four carbon atoms, and n is an integer from zero to 2, with the proviso that if n is 2, then said R_3 groups together contain no more than six carbon atoms;



IV

5

wherein

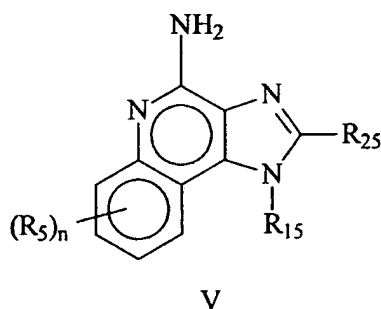
R_{14} is $-CHR_xR_y$ wherein R_y is hydrogen or a carbon-carbon bond, with the proviso that when R_y is hydrogen R_x is alkoxy of one to four carbon atoms, hydroxyalkoxy of one to four carbon atoms, 1-alkynyl of two to ten carbon atoms, tetrahydropyranyl, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to four carbon atoms, 2-, 3-, or 4-pyridyl, and with the further proviso that when R_y is a carbon-carbon bond R_y and R_x together form a tetrahydrofuranyl group optionally substituted with one or more substituents independently selected from the group consisting of hydroxy and hydroxyalkyl of one to four carbon atoms;

15

R_{24} is selected from the group consisting of hydrogen, alkyl of one to four carbon atoms, phenyl, and substituted phenyl wherein the substituent is selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms, and halogen; and

20

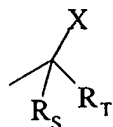
R_4 is selected from the group consisting of hydrogen, straight chain or branched chain alkoxy containing one to four carbon atoms, halogen, and straight chain or branched chain alkyl containing one to four carbon atoms, and n is an integer from 0 to 2, with the proviso that if n is 2 then said R_4 groups together contain no more than six carbon atoms;



wherein

5 R_{15} is selected from the group consisting of: hydrogen; straight chain or branched chain alkyl containing one to ten carbon atoms and substituted straight chain or branched chain alkyl containing one to ten carbon atoms, wherein the substituent is selected from the group consisting of cycloalkyl containing three to six carbon atoms and cycloalkyl containing three to six carbon atoms substituted by straight chain or branched chain alkyl containing one to four carbon atoms; straight chain or branched chain alkenyl containing
 10 two to ten carbon atoms and substituted straight chain or branched chain alkenyl containing two to ten carbon atoms, wherein the substituent is selected from the group consisting of cycloalkyl containing three to six carbon atoms and cycloalkyl containing three to six carbon atoms substituted by straight chain or branched chain alkyl containing one to four carbon atoms; hydroxyalkyl of one to six carbon atoms; alkoxyalkyl wherein
 15 the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to six carbon atoms; acyloxyalkyl wherein the acyloxy moiety is alkanoyloxy of two to four carbon atoms or benzoyloxy, and the alkyl moiety contains one to six carbon atoms; benzyl; (phenyl)ethyl; and phenyl; said benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected
 20 from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms, and halogen, with the proviso that when said benzene ring is substituted by two of said moieties, then the moieties together contain no more than six carbon atoms;

R₂₅ is



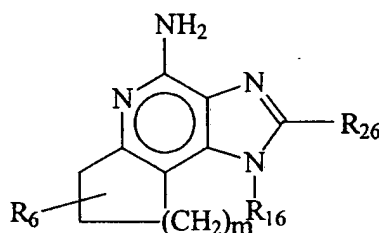
5 wherein

R_S and R_T are independently selected from the group consisting of hydrogen, alkyl of one to four carbon atoms, phenyl, and substituted phenyl wherein the substituent is selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms, and halogen;

10 X is selected from the group consisting of alkoxy containing one to four carbon atoms, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to four carbon atoms, hydroxyalkyl of one to four carbon atoms, haloalkyl of one to four carbon atoms, alkylamido wherein the alkyl group contains one to four carbon atoms, amino, substituted amino wherein the substituent is alkyl or
15 hydroxyalkyl of one to four carbon atoms, azido, chloro, hydroxy, 1-morpholino, 1-pyrrolidino, alkylthio of one to four carbon atoms; and

R₅ is selected from the group consisting of hydrogen, straight chain or branched chain alkoxy containing one to four carbon atoms, halogen, and straight chain or branched chain alkyl containing one to four carbon atoms, and n is an integer from 0 to 2, with the
20 proviso that if n is 2, then said R₅ groups together contain no more than six carbon atoms.

Preferred 6,7 fused cycloalkylimidazopyridine-4-amine IRM compounds are defined by Formula VI below:



VI

5 wherein m is 1, 2, or 3;

R_{16} is selected from the group consisting of hydrogen; cycloalkyl of three, four, or five carbon atoms; straight chain or branched chain alkyl containing one to ten carbon atoms and substituted straight chain or branched chain alkyl containing one to ten carbon atoms, wherein the substituent is selected from the group consisting of cycloalkyl
 10 containing three to six carbon atoms and cycloalkyl containing three to six carbon atoms substituted by straight chain or branched chain alkyl containing one to four carbon atoms; fluoro- or chloroalkyl containing from one to ten carbon atoms and one or more fluorine or chlorine atoms; straight chain or branched chain alkenyl containing two to ten carbon atoms and substituted straight chain or branched chain alkenyl containing two to ten
 15 carbon atoms, wherein the substituent is selected from the group consisting of cycloalkyl containing three to six carbon atoms and cycloalkyl containing three to six carbon atoms substituted by straight chain or branched chain alkyl containing one to four carbon atoms; hydroxyalkyl of one to six carbon atoms; alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to six carbon atoms;
 20 acyloxyalkyl wherein the acyloxy moiety is alkanoyloxy of two to four carbon atoms or benzoyloxy, and the alkyl moiety contains one to six carbon atoms, with the proviso that any such alkyl, substituted alkyl, alkenyl, substituted alkenyl, hydroxyalkyl, alkoxyalkyl, or acyloxyalkyl group does not have a fully carbon substituted carbon atom bonded directly to the nitrogen atom; benzyl; (phenyl)ethyl; and phenyl; said benzyl, (phenyl)ethyl
 25 or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to four carbon

atoms, alkoxy of one to four carbon atoms, and halogen, with the proviso that when said benzene ring is substituted by two of said moieties, then the moieties together contain no more than six carbon atoms;

and $-\text{CHR}_x\text{R}_y$

5 wherein

R_y is hydrogen or a carbon-carbon bond, with the proviso that when R_y is hydrogen R_x is alkoxy of one to four carbon atoms, hydroxyalkoxy of one to four carbon atoms, 1-alkynyl of two to ten carbon atoms, tetrahydropyranyl, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to four carbon
10 atoms, 2-, 3-, or 4-pyridyl, and with the further proviso that when R_y is a carbon-carbon bond R_y and R_x together form a tetrahydrofuranyl group optionally substituted with one or more substituents independently selected from the group consisting of hydroxy and hydroxyalkyl of one to four carbon atoms,

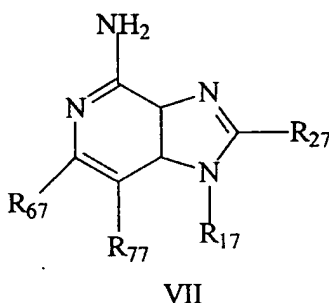
R_{26} is selected from the group consisting of hydrogen, straight chain or branched
15 chain alkyl containing one to eight carbon atoms, straight chain or branched chain hydroxyalkyl containing one to six carbon atoms, morpholinoalkyl wherein the alkyl moiety contains 1 to 4 carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by a moiety selected from the group consisting of methyl, methoxy, and halogen; and

20 $-\text{C}(\text{R}_S)(\text{R}_T)(\text{X})$ wherein R_S and R_T are independently selected from the group consisting of hydrogen, alkyl of one to four carbon atoms, phenyl, and substituted phenyl wherein the substituent is selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms, and halogen;

X is selected from the group consisting of alkoxy containing one to four carbon
25 atoms, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to four carbon atoms, haloalkyl of one to four carbon atoms, alkylamido wherein the alkyl group contains one to four carbon atoms, amino, substituted amino wherein the substituent is alkyl or hydroxyalkyl of one to four carbon atoms, azido, alkylthio of one to four carbon atoms, halogen, hydroxy, morpholino, and morpholinoalkyl
30 wherein the alkyl moiety contains one to four carbon atoms, and

R_6 is selected from the group consisting of hydrogen, fluoro, chloro, straight chain or branched chain alkyl containing one to four carbon atoms, and straight chain or branched chain fluoro- or chloroalkyl containing one to four carbon atoms and at least one fluorine or chlorine atom.

- 5 Preferred imidazopyridine-4-amine IRM compounds are defined by Formula VII below:



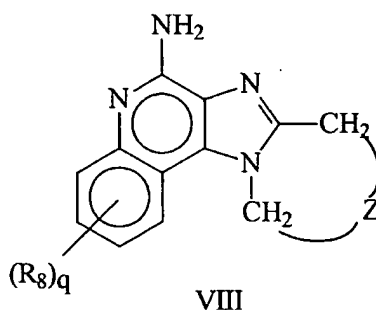
wherein

- 10 R_{17} is selected from the group consisting of hydrogen; $-CH_2R_w$ wherein R_w is selected from the group consisting of straight chain, branched chain, or cyclic alkyl containing one to ten carbon atoms, straight chain or branched chain alkenyl containing two to ten carbon atoms, straight chain or branched chain hydroxyalkyl containing one to six carbon atoms, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon
- 15 atoms and the alkyl moiety contains one to six carbon atoms, and phenylethyl; and $-CH=CR_ZR_Z$ wherein each R_Z is independently straight chain, branched chain, or cyclic alkyl of one to six carbon atoms;

- R_{27} is selected from the group consisting of hydrogen, straight chain or branched chain alkyl containing one to eight carbon atoms, straight chain or branched chain
- 20 hydroxyalkyl containing one to six carbon atoms, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to six carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by a moiety selected from the group consisting of methyl, methoxy, and halogen; and morpholinoalkyl wherein the alkyl moiety contains
- 25 one to four carbon atoms; and

R_{67} and R_{77} are independently selected from the group consisting of hydrogen and alkyl of one to five carbon atoms, with the proviso that R_{67} and R_{77} taken together contain no more than six carbon atoms, and with the further proviso that when R_{77} is hydrogen then R_{67} is other than hydrogen and R_{27} is other than hydrogen or morpholinoalkyl, and
 5 with the further proviso that when R_{67} is hydrogen then R_{77} and R_{27} are other than hydrogen.

Preferred 1,2-bridged imidazoquinoline-4-amine IRM compounds are defined by Formula VIII below:



10 wherein

Z is selected from the group consisting of:

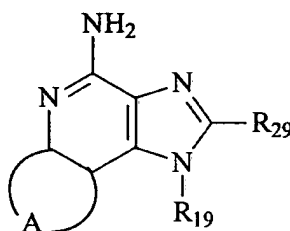
$-(CH_2)_p-$ wherein p is 1 to 4;

$-(CH_2)_a-C(R_D R_E)(CH_2)_b-$, wherein a and b are integers and a+b is 0 to 3, R_D is hydrogen or alkyl of one to four carbon atoms, and R_E is selected from the group
 15 consisting of alkyl of one to four carbon atoms, hydroxy, $-OR_F$ wherein R_F is alkyl of one to four carbon atoms, and $-NR_G R'_G$ wherein R_G and R'_G are independently hydrogen or alkyl of one to four carbon atoms; and

$-(CH_2)_a-(Y)-(CH_2)_b-$ wherein a and b are integers and a+b is 0 to 3, and Y is O, S, or $-NR_J-$ wherein R_J is hydrogen or alkyl of one to four carbon atoms;

20 and wherein q is 0 or 1 and R_8 is selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms, and halogen.

Preferred imidazonaphthyridine-4-amine and imidazotetrahydronaphthyridine-4-amine IRM compounds are defined by Formulas IX(a) and IX(b) below:



IX(a)

wherein

A is =N-CR=CR-CR=; =CR-N=CR-CR=; =CR-CR=N-CR=; or
=CR-CR=CR-N=;

5 R₁₉ is selected from the group consisting of:

- hydrogen;

-C₁₋₂₀ alkyl or C₂₋₂₀ alkenyl that is unsubstituted or substituted by one or more
substituents selected from the group consisting of:

-aryl;

10 -heteroaryl;

-heterocyclyl;

-O-C₁₋₂₀ alkyl,

-O-(C₁₋₂₀alkyl)₀₋₁-aryl;

-O-(C₁₋₂₀alkyl)₀₋₁-heteroaryl;

15 -O-(C₁₋₂₀alkyl)₀₋₁-heterocyclyl;

-C₁₋₂₀ alkoxycarbonyl;

-S(O)₀₋₂-C₁₋₂₀ alkyl;

-S(O)₀₋₂-(C₁₋₂₀ alkyl)₀₋₁-aryl;

-S(O)₀₋₂-(C₁₋₂₀ alkyl)₀₋₁-heteroaryl;

20 -S(O)₀₋₂-(C₁₋₂₀ alkyl)₀₋₁-heterocyclyl;

-N(R₃₉)₂;

-N₃;

oxo;

-halogen;

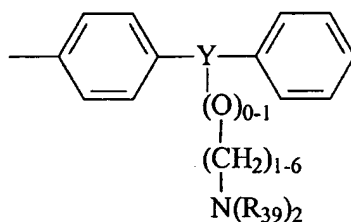
25 -NO₂;

-OH; and

-SH; and

5 $-C_{1-20}$ alkyl- NR_{39} -Q-X- R_{49} or $-C_{2-20}$ alkenyl- NR_{39} -Q-X- R_{49} wherein Q is $-CO-$ or $-SO_2-$; X is a bond, $-O-$ or $-NR_{39}-$ and R_{49} is aryl; heteroaryl; heterocyclyl; or $-C_{1-20}$ alkyl or C_{2-20} alkenyl that is unsubstituted or substituted by one or more substituents selected from the group consisting of:

10 -aryl;
 -heteroaryl;
 -heterocyclyl;
 $-O-C_{1-20}$ alkyl,
 $-O-(C_{1-20}alkyl)_{0-1}-aryl$;
 $-O-(C_{1-20}alkyl)_{0-1}-heteroaryl$;
 $-O-(C_{1-20}alkyl)_{0-1}-heterocyclyl$;
 15 $-C_{1-20}$ alkoxycarbonyl;
 $-S(O)_{0-2}-C_{1-20}$ alkyl;
 $-S(O)_{0-2}-(C_{1-20}alkyl)_{0-1}-aryl$;
 $-S(O)_{0-2}-(C_{1-20}alkyl)_{0-1}-heteroaryl$;
 $-S(O)_{0-2}-(C_{1-20}alkyl)_{0-1}-heterocyclyl$;
 20 $-N(R_{39})_2$;
 $-NR_{39}-CO-O-C_{1-20}alkyl$;
 $-N_3$;
 oxo;
 -halogen;
 25 $-NO_2$;
 -OH; and
 -SH; or R_{49} is



wherein Y is -N- or -CR-;

R₂₉ is selected from the group consisting of:

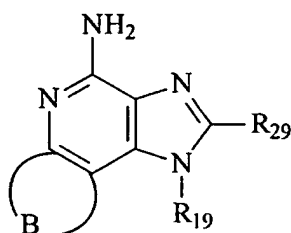
- hydrogen;
- 5 -C₁₋₁₀ alkyl;
- C₂₋₁₀ alkenyl;
- aryl;
- C₁₋₁₀ alkyl -O-C₁₋₁₀-alkyl;
- C₁₋₁₀ alkyl-O-C₂₋₁₀ alkenyl; and
- 10 -C₁₋₁₀ alkyl or C₂₋₁₀ alkenyl substituted by one or more substituents selected from

the group consisting of:

- OH;
- halogen;
- N(R₃₉)₂;
- 15 -CO-N(R₃₉)₂;
- CO-C₁₋₁₀ alkyl;
- N₃;
- aryl;
- heteroaryl;
- 20 -heterocyclyl;
- CO-aryl; and
- CO-heteroaryl;

each R₃₉ is independently selected from the group consisting of hydrogen and C₁₋₁₀ alkyl; and

- 25 each R is independently selected from the group consisting of hydrogen,
- C₁₋₁₀ alkyl, C₁₋₁₀ alkoxy, halogen and trifluoromethyl,



IX(b)

wherein

B is $-\text{NR}-\text{C}(\text{R})_2-\text{C}(\text{R})_2-\text{C}(\text{R})_2-$; $-\text{C}(\text{R})_2-\text{NR}-\text{C}(\text{R})_2-\text{C}(\text{R})_2-$;

5 $-\text{C}(\text{R})_2-\text{C}(\text{R})_2-\text{NR}-\text{C}(\text{R})_2-$ or $-\text{C}(\text{R})_2-\text{C}(\text{R})_2-\text{C}(\text{R})_2-\text{NR}-$;

R_{19} is selected from the group consisting of:

- hydrogen;

$-\text{C}_{1-20}$ alkyl or C_{2-20} alkenyl that is unsubstituted or substituted by one or more
substituents selected from the group consisting of:

10

-aryl;

-heteroaryl;

-heterocyclyl;

$-\text{O}-\text{C}_{1-20}$ alkyl;

$-\text{O}-(\text{C}_{1-20}\text{alkyl})_{0-1}-\text{aryl}$;

15

$-\text{O}-(\text{C}_{1-20}\text{alkyl})_{0-1}-\text{heteroaryl}$;

$-\text{O}-(\text{C}_{1-20}\text{alkyl})_{0-1}-\text{heterocyclyl}$;

$-\text{C}_{1-20}$ alkoxycarbonyl;

$-\text{S}(\text{O})_{0-2}-\text{C}_{1-20}$ alkyl;

$-\text{S}(\text{O})_{0-2}-(\text{C}_{1-20}\text{alkyl})_{0-1}-\text{aryl}$;

20

$-\text{S}(\text{O})_{0-2}-(\text{C}_{1-20}\text{alkyl})_{0-1}-\text{heteroaryl}$;

$-\text{S}(\text{O})_{0-2}-(\text{C}_{1-20}\text{alkyl})_{0-1}-\text{heterocyclyl}$;

$-\text{N}(\text{R}_{39})_2$;

$-\text{N}_3$;

oxo;

25

-halogen;

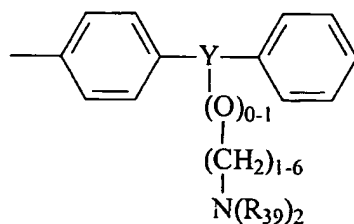
$-\text{NO}_2$;

-OH; and

-SH; and

5 -C₁₋₂₀ alkyl-NR₃₉-Q-X-R₄₉ or -C₂₋₂₀ alkenyl-NR₃₉-Q-X-R₄₉ wherein Q is -CO- or -SO₂-; X is a bond, -O- or -NR₃₉- and R₄₉ is aryl; heteroaryl; heterocyclyl; or -C₁₋₂₀ alkyl or C₂₋₂₀ alkenyl that is unsubstituted or substituted by one or more substituents selected from the group consisting of:

10 -aryl;
-heteroaryl;
-heterocyclyl;
-O-C₁₋₂₀ alkyl,
-O-(C₁₋₂₀alkyl)₀₋₁-aryl;
-O-(C₁₋₂₀alkyl)₀₋₁-heteroaryl;
-O-(C₁₋₂₀alkyl)₀₋₁-heterocyclyl;
15 -C₁₋₂₀ alkoxycarbonyl;
-S(O)₀₋₂ -C₁₋₂₀ alkyl;
-S(O)₀₋₂ -(C₁₋₂₀ alkyl)₀₋₁-aryl;
-S(O)₀₋₂ -(C₁₋₂₀ alkyl)₀₋₁-heteroaryl;
-S(O)₀₋₂ -(C₁₋₂₀ alkyl)₀₋₁-heterocyclyl;
20 -N(R₃₉)₂;
-NR₃₉-CO-O-C₁₋₂₀alkyl;
-N₃;
oxo;
-halogen;
25 -NO₂;
-OH; and
-SH; or R₄₉ is



wherein Y is -N- or -CR-;

R₂₉ is selected from the group consisting of:

- hydrogen;
- C₁₋₁₀ alkyl;
- 5 -C₂₋₁₀ alkenyl;
- aryl
- C₁₋₁₀ alkyl -O-C₁₋₁₀-alkyl;
- C₁₋₁₀ alkyl-O-C₂₋₁₀ alkenyl; and
- C₁₋₁₀ alkyl or C₂₋₁₀ alkenyl substituted by one or more substituents selected from
- 10 the group consisting of:
- OH;
- halogen;
- N(R₃₉)₂;
- CO-N(R₃₆)₂;
- 15 -CO-C₁₋₁₀ alkyl;
- N₃;
- aryl;
- heteroaryl;
- heterocyclyl;
- 20 -CO-aryl; and
- CO-heteroaryl;

each R₃₉ is independently selected from the group consisting of hydrogen and C₁₋₁₀ alkyl; and

each R is independently selected from the group consisting of hydrogen, C₁₋₁₀ alkyl, C₁₋₁₀ alkoxy, halogen and trifluoromethyl.

The substituents R₁₁ - R₁₉ above are generally designated "1-substituents", as they are located at the 1-position of the various ring systems. Preferred 1-substituents include alkyl containing one to six carbon atoms and hydroxyalkyl containing one to six carbon atoms. More preferably the 1- substituent is 2-methylpropyl or 2-hydroxy-2-methylpropyl.

The substituents R₂₁ - R₂₉ above are generally designated "2-substituents", due to their placement at the 2-position of the various ring systems. Preferred 2-substituents

include hydrogen, alkyl of one to six carbon atoms, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to four carbon atoms, and hydroxyalkyl of one to four carbon atoms. More preferably the 2-substituent is hydrogen, methyl, butyl, hydroxymethyl, ethoxymethyl or methoxyethyl.

5 In instances where n can be zero, one, or two, n is preferably zero or one.

As used herein, the terms "alkyl", "alkenyl", and the prefix "-alk" are inclusive of both straight chain and branched chain groups and of cyclic groups, i.e. cycloalkyl and cycloalkenyl. These cyclic groups can be monocyclic or polycyclic and preferably have from 3 to 10 ring carbon atoms. Exemplary cyclic groups include cyclopropyl,
10 cyclopentyl, cyclohexyl and adamantyl. Alkyl and alkenyl groups contain from 1 to 10 (or 2 to 10) carbon atoms unless otherwise specified.

The term "aryl" as used herein includes carbocyclic aromatic rings or ring systems. Examples of aryl groups include phenyl, naphthyl, biphenyl, fluorenyl and indenyl. The term "heteroaryl" includes aromatic rings or ring systems that contain at least one ring
15 hetero atom (e.g. O, S, N). Suitable heteroaryl groups include furyl, thienyl, pyridyl, quinolinyl, tetrazolyl, imidazolyl, and so on.

"Heterocyclyl" includes non-aromatic rings or ring systems that contain at least one ring hetero atom (e.g. O, S, N). Exemplary heterocyclic groups include pyrrolidinyl, tetrahydrofuranyl, morpholinyl, thiazolidinyl, imidazolidinyl and the like.

20 The aryl, heteroaryl and heterocyclyl groups may be unsubstituted or substituted by one or more substituents selected from the group consisting of C₁₋₂₀ alkyl, hydroxy, halogen, N(R₁₀)₂ where each R₁₀ is independently selected from the group consisting of hydrogen, C₁₋₁₀ alkyl, NO₂, C₁₋₂₀ alkoxy, C₁₋₂₀ alkylthio, trihalomethyl, C₁₋₂₀ acyl, arylcarbonyl, heteroarylcarbonyl, (C₁₋₁₀alkyl)₀₋₁-aryl, (C₁₋₁₀alkyl)₀₋₁-heteroaryl, nitrile,
25 C₁₋₂₀ alkoxycarbonyl, oxo, arylalkyl wherein the alkyl group has from 1 to 10 carbon atoms, and heteroarylalkyl wherein the alkyl group has from 1 to 10 carbon atoms.

The invention is inclusive of the compounds described herein in any of their pharmaceutically acceptable forms, including salts, isomers such as diastereomers and enantiomers, solvates, polymorphs, and the like.

30 Of the foregoing IRM compounds, those having the imidazoquinoline structure are preferred. In particular, imidazoquinoline-4-amine compounds of formulas I and V are

preferred. The compounds 4-amino-2-ethoxymethyl- α,α -dimethyl-1H-imidazo[4,5-c]quinolin-1-ethanol and 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine are especially preferred.

The IRM compounds useful in the methods of the invention can be prepared using methods that are known in the art, as seen for example in U.S. Patent Nos. 4,689,338, 5,389,640, 5,268,376, 4,929,624, 5,266,575, 5,352,784, 5,494,916, 5,482,936, 5,346,905, 5,395,937, 5,756,747, 4,988,815, 5,175,296, 5,741,908, 5,367,076, 5,693,811 and 5,525,612, and in copending U.S. Patent Application Serial No. 09/210,114 all of which are incorporated by reference herein.

Maturation of Dendritic Cells

The IRM compounds described above have been found to induce the maturation of DC *ex vivo*. In general, mature DC display properties such as cytokine secretion, the expression of particular cell surface markers, and an enhanced ability to stimulate T-cells.

Dendritic cells that can be matured using the method of the invention can be obtained from any source, which sources can be readily determined by those of skill in the art. For example, the immature DC can be obtained by isolating the DC from tissues such as blood, spleen, bone marrow, skin (e.g., Langerhans cells) and the like or by inducing the differentiation of monocytes or stem cells using methods known in the art. A preferred method of obtaining DC comprises the cytokine-induced differentiation of human peripheral blood mononuclear cells. This method has been described, for example by Romani et al., *J. Immunol. Methods* 196:137 (1996) and Bender et al., *J. Immunol. Methods* 196:121 (1996). A particularly preferred method comprises culturing CD14⁺ peripheral blood monocytes with GM-CSF and IL-4 using the method described by Romani, *supra*.

The DC thus obtained will be in an immature state, generally possessing a high capability for antigen capture and processing, but relatively low T-cell stimulatory capacity. To acquire optimal T-cell stimulating capacity, the DC must be in a stable, mature state. Mature DC can be identified by a number of properties, including their expression of the cell surface marker CD83 and by the behavior displayed during the mixed lymphocyte reaction. In this reaction mature DC will cause increased proliferation

of naïve allogeneic T-cells and/or increased production of dendritic cell cytokines. Preferably, the mature DC will induce at least a two- fold increase in the proliferation of naïve allogeneic T-cells and/or will display at least a three- fold increase in the production of dendritic cell cytokines, particularly IL-12 and TNF- α , as compared to DC that have
5 been obtained from the same source but have not been contacted with any exogenous stimuli ("immature DC"). While immature DC may display some of the properties described above, they display them to a much lesser extent than DC which have been matured by exposure to exogenous stimuli such as an imidazoquinoline type IRM compound. The mature DC should be stable and not revert to their immature state, as the
10 immature DC are much less potent stimulators of T-cell activity.

The method of the invention comprises the maturation of DC by stimulating the DC with an imidazoquinoline type IRM in an amount and for a time sufficient to cause the DC to mature. It is understood that the DC are incubated in a tissue culture medium under conditions readily determinable to those of skill in the art. The specific amount of IRM
15 used and the time of exposure will vary according to a number of factors that will be appreciated by those of skill in the art, including the origin of the DC to be matured, the potency and other characteristics of the IRM compound used, and so on. However, it is currently preferred that the IRM be used at a concentration of about 0.1 to about 10 μ g/ml, preferably about 0.5 to about 2.0 μ g/ml. The IRM compound is solubilized before being
20 added to the DC containing medium, preferably in water or a physiological buffer. However, if necessary the compound can be solubilized in a small amount of an organic solvent such as DMSO and then diluted or added directly to the DC containing medium.

The DC are stimulated by the IRM compound for a sufficient amount of time to allow the DC to become fully mature. This can be determined by periodically
25 withdrawing samples of the DC containing medium and assaying for one of the above described properties, such as secretion of dendritic cell cytokines. In general, the DC can be said to be fully mature when the measured property has attained its maximal level and is no longer increasing with time. Although the time of exposure will vary according to factors understood by those of skill in the art (including but not limited to the origin of the
30 DC, the concentration and potency of the IRM, and so on), in general approximately 16 to 24 hours of stimulation are required for the DC to become fully mature.

Dendritic cells that have been matured by exposure to one or more imidazoquinoline type IRMs express CD83 and display enhanced expression of CD80, CD86 and CD40. In addition, IRM matured DC secrete a number of cytokines, particularly pro-inflammatory cytokines such as TNF- α , IFN- α , IL-6, IL-1, IL-12 p40.

5

Use of IRM Matured Dendritic Cells

Dendritic cells that have been matured by exposure to imidazoquinoline type IRMs have enhanced antigen presenting ability as compared to immature DC and can be used in a variety of ways to enhance the immune response of a subject. For example, the mature DC can be injected directly into a patient. In this case, the DC are preferably monocyte derived DC wherein the monocytes have been obtained from the same patient.

The DC can also be used in a number of immunotherapies. Examples of such therapies include *ex vivo* cell transplantation therapies for treating disorders of the immune system, such as AIDS; the *ex vivo* expansion of T-cells, particularly antigen specific T-cells which can then be used to treat disorders characterized by deterioration of the immune system; the generation of monoclonal antibodies that recognize DC-specific markers; the preparation of antigen activated DC according to methods known in the art; and development of vaccines and vaccine adjuvants.

Preferred uses of DC that have been matured by exposure to one or more imidazoquinoline type IRMs include those that make use of antigen activated DC and/or DC modified antigens. The antigen activated DC, or cellular adjuvants, of the invention are generally prepared by exposing DC matured according to the method of the invention to an antigen. The antigen may be protein, carbohydrate or nucleic acid in nature and may be derived from any suitable source, including neoplastic cells (e.g., tumor cells) and infectious agents (e.g., bacterium, virus, yeast, parasite). Alternatively, the antigen can be derived by recombinant means.

The cellular adjuvant of the invention can be used in the treatment of diseases. For example, cellular adjuvants prepared by exposing the mature DC to tumor derived antigens can be administered to a patient, thereby provoking an anti-tumor immune response in the patient. Similarly, infectious diseases can be treated by administering to the patient

cellular adjuvants prepared by exposing the DC to antigens derived from the infectious agent.

Dendritic cells that have been matured by the method of the invention produce cytokines such as IL-12 and IFN- α that favor the generation of Th1 immune responses.

5 The ability to bias the immune response towards the Th1, as opposed to the Th2, response, can provide a means for treatment of Th2 mediated diseases. Examples of such diseases include asthma; allergic rhinitis; systemic lupus erythematosus; eczema; atopic dermatitis Ommen's syndrome (hyperseosinophilia syndrome); certain parasitic infections such as cutaneous and systemic leishmaniasis, toxoplasma infection and trypanosome infection; 10 certain fungal infections, for example candidiasis and histoplasmosis; and certain intracellular bacterial infections such as leprosy and tuberculosis.

Experimental

Materials and Methods

15 *Culture Medium.* Complete RPMI (cRPMI) medium was used throughout this study. cRPMI consists of RPMI 1640 with 25 mM HEPES (Life Technologies, Gaithersburg, MD) supplemented with 10% heat inactivated FCS (Hyclone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1 mM L-glutamine and 50 μ g/ml gentamicin sulphate (Life Technologies).

20 *Reagents.* Peripheral blood derived CD14⁺ cells were differentiated into DC using recombinant human GM-CSF and recombinant human IL-4 at 800 U/ml and 25 ng/ml, respectively (R&D Corporation, Minneapolis, MN), as described by Romani and Bender, supra. Tetanus toxoid (Calbiochem, La Jolla, CA) was solubilized in cRPMI and used at 10 μ g/ml. The compound R-848 (S-28463), 4-amino-2-ethoxymethyl- α,α -dimethyl-1H-imidazo[4,5-c]quinoline-1-ethanol, M.W. = 314.4, was prepared by 3M Pharmaceuticals, 25 St. Paul, MN. For cell culture studies, the HCl salt was dissolved in pyrogen-free, sterile water and stored as a stock solution at 4°C for up to 4 months. Endotoxin levels were below the detectable level [1 pg/mg] in the Limulus amoebocyte assay. A stock solution of bacterial LPS from *Escherichia coli* 055:B5 (Sigma Chemical, St. Louis, MO) was 30 dissolved at 1 mg/ml in pyrogen-free water and stored at 4°C until use.

Generation of Monocyte-Derived Dendritic Cells (MO-DC). PBMC were isolated with Histopaque HybriMax -1077 density gradient (Sigma) from healthy volunteers after obtaining informed consent. CD14⁺ cells were purified by positive selection using CD14⁺ microbeads in conjunction with the MiniMACS system (Miltenyi Biotech, Auburn, CA) by following the manufacturer's instructions. Purity, as assessed by flow cytometry, was greater than 90%. The CD14⁺ cells were cultured at $2-5 \times 10^6$ cells per 3 ml cRPMI in 6-well plates (Costar, Cambridge, MA) with 800 U/ml GM-CSF and 25 ng/ml IL-4 as previously described by Romani and Bender, supra. Fresh medium containing GM-CSF and IL-4 was added every three days. MO-DC were routinely used between days 7 and 8 of culture. As a control, depleted lymphocytes were cultured in the same fashion.

In Vitro MO-DC Stimulation. MO-DC were stimulated with 0.1 to 8 µg/ml R-848 (1 µg/ml = 3.2 µM) or 1 µg/ml LPS for 1-96 hours. Cells were subsequently analyzed by flow cytometry for the expression of various cell surface markers, and the cell culture supernatants were analyzed for various cytokines and chemokines by ELISA.

Cell Surface and Intracellular Flow Cytometry. Evaluation of cell surface marker expression was performed by flow cytometric analysis using the following monoclonal antibodies: FITC-conjugated CD1a, clone NA1/34 HLK (Accurate Chemical, Westbury, NY); PE-conjugated CD14, clone MφP9, PE-conjugated CD80, clone L307.4, PE- and FITC-conjugated HLA-DR, clone L243, PE- and FITC-conjugated γ1/γ2a isotype control, clones X40 and X39 (all from Becton Dickinson, Mountain View, CA); PE-conjugated CD40, clone EA-5 (Biosource International, Camarillo, CA); PE-conjugated CD83, clone HB15a, PE- and FITC-conjugated γ1/γ1 isotype control, clone 679.1Mc7 (Immunotech, Marseille, France), PE-conjugated CD86, clone 2331 (Pharmingen, San Diego, CA). Cells (5×10^5) were incubated for 15 minutes incubation at 4°C with purified IgD (Becton Dickinson) to block non-specific binding, and then the cells were stained for 30 minutes with the antibodies at 4°C in PBS containing 10% FCS and 0.1% sodium azide. After washing in PBS, the cells were analyzed using a FACScan flow cytometer and Cell Quest software (Becton Dickinson).

Allogeneic Lymphocyte Activation. T-cells were isolated using T-Cell Purification Columns according to manufacturer's specifications (R&D Systems, Minneapolis, MN). Allogeneic MO-DC stimulator cells were pulsed for various times with medium alone, R-

848, or LPS for 1, 6 or 24 hrs and then washed and treated with 50 µg/ml mitomycin C (Sigma) for 20 minutes at 37°C. Dendritic cells were subsequently washed, resuspended in cRPMI and added at various concentrations ($1-32 \times 10^3$ per well) to purified responder T-cells (1×10^5 per well) in 96-well flat-bottomed microtiter plates (BD Labware) in a total volume of 200 µl. Triplicate cultures were maintained at 37°C for 96 hours after which time cell proliferation was assessed by incorporation of [³H]-thymidine ([³H]- TdR) (Amersham, Arlington Heights, IL). Each well received 1 µCi [³H]TdR and was harvested 18 hours later. Results are presented as mean CPM ± SEM of triplicate wells .

Supernatants were collected from the same cultures prior to pulsing with [³H]TdR and analyzed for IFN-γ, IL-5 and IL-2.

Autologous T Cell Activation. Autologous T cells and R-848-treated MO-DC were prepared as described for allogeneic T cell stimulation. MO-DC were cultured with R-848 [2 µg/ml] and tetanus toxoid [10 µg/ml] for 24 hours. The MO-DC were washed and cultured at graded doses with PBMC-derived CD3⁺ T cells for 7 days. Cell proliferation and analysis were determined as described. Supernatants were also collected from the same cultures prior to pulsing with [³H]TdR and analyzed for IFN-γ and IL-5.

Cytokine Analysis. Cytokine levels were measured by ELISA. Human TNF-α, IL-12 (p40/p70), IFNγ, IL-4 and IL-2 kits were purchased from Genzyme (Cambridge, MA). Human IL-6 kits were obtained from Biosource International (Camarillo, CA). Human IL-5, IL-8, MIP-1α, MCP-1 and RANTES were purchased from R&D Systems. All ELISA were run according to manufacturer's specifications. IFN levels were measured by bioassay (40). IFN-α and IFN-β specific antibodies were used to determine which type I IFN was present in the MO-DC supernatants. Results for all ELISAs are presented in pg/ml, whereas IFN results are presented in U/ml.

Statistical Analysis. Data were analyzed using a paired Student's t-test, and the results were considered statistically significant if $p \leq 0.05$.

To assess the maturation potential of R-848 on DC, MO-DC were treated with R-848 [0.1-8 µg/ml] or LPS [1 µg/ml] for 24 hours, and cell surface CD83 and CD86 expression were analyzed by flow cytometry on the DC (gated) population as defined by the forward scatter/side scatter characteristics (Figure 1A). The results in Figure 1B demonstrate that R-848 enhances the expression of CD83 and CD86 on MO-DC as

compared to unstimulated (vehicle) cells. There was no increase in either CD83 or CD86 cell surface expression with 0.1 $\mu\text{g/ml}$ R848. Enhanced CD86 expression is evident with 0.4, 2 and 8 $\mu\text{g/ml}$ R-848. Enhanced cell surface expression of CD83 is seen at 2 and 8 $\mu\text{g/ml}$ R-848. Both CD83 and CD86 cell surface expression are also enhanced with LPS, which has been shown to enhance the expression of these molecules on DC. Figure 1C represents the quantitative CD83 and CD86 cell surface expression in mean fluorescence intensity (MFI) of R-848 treated MO-DC. R-848 induces an increase of both CD83 and CD86 expression in a dose dependent manner, with CD86 expression increasing between 0.1-0.4 $\mu\text{g/ml}$ R-848. CD83 expression is significantly increased between 0.4-2 $\mu\text{g/ml}$ R-848. Maximal increases in both CD83 and CD86 expression are generated with 2 $\mu\text{g/ml}$ R-848, which corresponds to an average increase of approximately 3- to 4-fold for both CD80 and CD86. Comparatively, maximal CD83 and CD86 cell surface expression induced with R-848 was equivalent to that induced by LPS. Both the relative cell number and MFI data correlate indicating an increased number of cells expressing these antigens in response to R-848.

In addition to CD83 and CD86, other cell surface molecules indicative of DC maturation were also examined by flow cytometry. MO-DC were cultured with 2 $\mu\text{g/ml}$ R-848 for 24 hours, which gave maximal CD83 and CD86 expression as shown in Figure 1. The cells were stained for cell surface expression of CD1a, CD80, CD83, CD86, CD40 and HLA-DR. Figure 2A demonstrates that R-848 also enhances the expression of CD80 and CD40, in addition to CD83 and CD86, as compared to vehicle controls. Figures 2B and 2C represent the quantitative differences in cell surface molecule expression. Consistent with the increase in CD83 and CD86 expression, R-848 treatment also induces a 2-fold increase in CD80 and CD40 expression over the vehicle treated MO-DC. Although R-848-induces an increase in cell surface HLA-DR expression (Figures 2A and 2C), the increase is not quantitatively significant. Similarly, the R-848-induced decrease in CD1a expression is not statistically significant. These trends in HLA-DR and CD1a expression following R-848 stimulation were seen in all experiments, and in some experiments, the differences were statistical significant between R-848 and vehicle treated cells. LPS used at 1 $\mu\text{g/ml}$ enhanced cell surface expression of CD40, CD80, CD86 and CD83 to similar levels induced by R-848 (data not shown). The results in Figures 1 and 2

demonstrate that R-848 induces MO-DC maturation as defined by increased CD83, CD80, CD86 and CD40 expression. These DC maturation markers were also examined after 48, 72 and 96 hour stimulation with R-848, and maximal DC maturation marker expression was obtained after 24 hours in culture with 2 µg/ml R-848.

5 *R-848 Induces the Secretion of Pro-inflammatory Cytokines and Chemokines from Monocyte-Derived Dendritic Cells*

DC maturation results in the production of various cytokines and chemokines. In addition, numerous cytokines produced by mature DC such as TNF-α and IL-12 can induce or enhance DC maturation. Therefore, we tested if R-848 induces MO-DC
10 cytokine and chemokine secretion characteristic of DC maturation. MO-DC were cultured with various concentrations of R-848 for 24 hours as in Figures 1 and 2. The supernatants were analyzed for secreted cytokines and chemokines by ELISA or by bioassay. The results in Table I indicate that MO-DC treated with R-848 produce significantly more TNF-α, IL-6, IL-12, IL-8, MIP-1α and IFN-α as compared to the vehicle control.
15 Although statistically significant levels of all the tested cytokines are obtained with 2 µg/ml R-848, IL-6, IL-8 and IL-12 appear to be induced with R-848 between 0.1-0.4 µg/ml, but the levels are not statistically different than those produced by the vehicle-treated MO-DC. MCP-1 levels were increased with 0.1-8 µg/ml R-848, but not significantly different from the levels produced by the control cells. Neutralizing IFN-α
20 inhibited greater than 95% of the bioactivity, indicating that the IFN induced by R-848 was IFN-α. Similar to R-848, LPS significantly enhanced TNF-α, IL-6, IL-12, MIP-1α and IFN-α as compared to the vehicle control group. The maximal cytokine and chemokine levels induced by LPS are comparable to the maximal levels induced by R-848.

The length of time MO-DC need to be in contact with R-848 for maturation to
25 occur was determined by pulsing the cells with R-848 for various periods of time. Culture supernatants were analyzed for cytokine secretion after various treatment times with R-848 or LPS. TNF-α and IL-12 secretion were used as markers of DC maturation on the basis of the results in Table I and on previous studies. First, MO-DC were cultured with 2 µg/ml R-848 or 1 µg/ml LPS for 1, 6 or 24 hours, and the supernatants were then analyzed
30 for cytokine secretion immediately post culture (Table II, Groups I, II and V). The results

in Table II demonstrate that MO-DC produce minimal amounts of TNF- α and IL-12 after one hour stimulation with R-848. A significant increase in TNF- α and IL-12 protein is detected in the supernatants following 6 hour stimulation with R-848. R-848 treatment for 24 hours also induces a significant increase in TNF- α and IL-12 secretion. The LPS groups produced both TNF- α and IL-12 with the same kinetics as the R-848-treated groups, except LPS induced approximately 2-fold more TNF- α than was induced by R-848. LPS treated MO-DC produced approximately 5-fold more IL-12 than R-848 treated MO-DC.

The results in Table II indicate that MO-DC require greater than one hour stimulation with either R-848 or LPS in order to secrete significant levels of TNF- α and IL-12. Maximal TNF- α secretion is achieved between one and six hours stimulation, and maximal IL-12 secretion requires between six and twenty four hours stimulation with either R-848 or LPS.

In addition to TNF- α and IL-12 production, cell surface markers of DC maturation were also examined by flow cytometry following R-848 treatment for various times in order to determine the length of time MO-DC need to be in culture with R-848 for optimal maturation marker expression. MO-DC pulsed for one hour with 2 μ g/ml R-848 or 1 μ g/ml LPS, and then stained for DC maturation markers, did not show enhanced expression of CD83, CD80, CD86, CD40 or HLA-DR. MO-DC pulsed for 6 hours with R-848 and stained immediately for maturation markers show a significant increase in CD83 but not CD80, CD86, CD40 or HLA-DR (Fig. 3A and 3B). Although CD40, CD86 and HLA-DR expression are elevated in the R-848 treated group following 6 hours in culture, the differences are not statistically significant as compared to the medium control. Similar to R-848 treated MO-DC, LPS treated MO-DC showed enhanced CD83 expression, but no change in CD40, CD80, CD86 and HLA-DR expression.

MO-DC were pulsed for 1 or 6 hours with 2 μ g/ml R-848 or 1 μ g/ml LPS, washed free of stimulus, and then re-cultured for an additional 23 hours (1 hour pulse) or 18 hours (6 hour pulse) before cell surface DC maturation marker determination. MO-DC pulsed for one hour with 2 μ g/ml R-848 or 1 μ g/ml LPS did not show enhanced expression of CD83, CD80, CD86, CD40 or HLA-DR after 24 hours in culture. MO-DC pulsed for 6 hours with R-848 show a significant increase in CD83 and CD40 expression, but not

CD80, CD86 or HLA-DR after 24 hours in culture (Fig. 3C and 3D). The expression of CD86 and HLA-DR markers are elevated above, but not statistically different, than the medium control group. Comparable results were obtained with similarly cultured LPS-stimulated MO-DC.

5 *Allogeneic T cell Proliferation and T cell Cytokine Secretion are Increased by R-848-treated Monocyte-Derived Dendritic Cells*

To determine if the functional features of DC were altered by imidazoquinoline-treatment, R-848-stimulated MO-DC were tested in a primary MLR. MO-DC were treated with 0.1-8 µg/ml R-848 or 1 µg/ml LPS. After 24 hours, the MO-DC were washed free of
10 stimulating agent and cultured with allogeneic CD3-enriched peripheral blood T cells for 96 hours, whereby cell proliferation was assessed by [³H]thymidine incorporation. The results in Figure 4A demonstrate that R-848-treated MO-DC were more efficacious stimulators of allogeneic T cell proliferation than vehicle-treated cells, and R-848-treated cells were as effective as LPS-stimulated cells. A significant difference in T cell
15 proliferation is seen when MO-DC are treated with 2 or 8 µg/ml R-848 as compared to vehicle-treated MO-DC.

MLR supernatants were analyzed for T cell cytokines following 96 hours of culture. R-848-treated MO-DC enhance IL-2, IL-5 and IFN-γ secretion from allogeneic T cells as compared to the vehicle control group (Figure 4B-4D). Concordant with the MLR
20 proliferation results in Figure 4A, a significant 2- to 3-fold enhancement of IL-2, IL-5 and IFN-γ production was induced by cultures containing MO-DC treated with 2 and 8 µg/ml R-848 as compared to the untreated MO-DC cultures. T cell cytokines induced by R-848-stimulated MO-DC were equivalent to cytokine levels induced by LPS-stimulated MO-DC. IL-2, IL-5 and IFN-γ production require MO-DC cultured with T cells, because
25 cultures containing only MO-DC or only T cells did not produce detectable levels of IL-2, IL-5 or IFN-γ. Additionally, T cells cultured in the presence of R-848, without added MO-DC, do not produce IL-2, IL-5 or IFN-γ. These data indicate that R-848 enhances DC function equivalent to that induced by LPS. Although maximal proliferation was induced by MO-DC that were pulsed for 24 hours with R-848, MO-DC treated for 6 hours with R-
30 848 also significantly enhanced allogeneic T cell proliferation as compared to untreated MO-DC. When MO-DC were treated for less than 6 hours with R-848, allogeneic T cell

proliferation was not significantly increased as compared to the untreated MO-DC controls.

Autologous T cell Proliferation and T cell Cytokine Secretion are Increased by R-848-treated Monocyte-Derived Dendritic Cells

5 The effect of R-848 on MO-DC function was also tested in an autologous (syngeneic) anamnestic response to tetanus toxoid. MO-DC were treated with 2 µg/ml R-848 and 10 µg/ml tetanus toxoid for 24 hours. The MO-DC were washed free of compound and antigen and then cultured with syngeneic CD3-enriched peripheral blood T cells for 7 days at which time proliferation was assessed by [³H]thymidine incorporation. 10 The results in Figure 5A and 5B indicate that tetanus toxoid-treated MO-DC and untreated MO-DC induced the same amount of syngeneic T cell proliferation. However, R-848-treated MO-DC increased T cell proliferation by 2-to 3-fold as compared to the MO-DC that were not treated with R-848. Cytokine secretion was also analyzed from the autologous MO-DC/T cell system. IFN-γ secretion was only detected in the supernatants 15 that contained MO-DC treated with both R-848 and tetanus toxoid (Figures 5C and 5D). MO-DC treated with both R-848 and tetanus toxoid produced 4- to 11-fold more IFN-γ than MO-DC cultured only with the tetanus toxoid antigen. IL-5 was not detected in any of the same culture supernatants containing IFN-γ. The data in Figure 5 indicate that memory T cell IFN-γ secretion, but not proliferation, is enhanced by R-848-treated MO- 20 DC.

Detailed Description of the Drawings

Figure 1. The immune response modifier R-848 enhances cell surface expression of CD83 and CD86 on monocyte-derived dendritic cells (MO-DC). MO-DC were 25 generated in vitro from CD14⁺ PBMC as described in Materials and Methods. MO-DC (2 x 10⁵) were stimulated with 0.1-8 µg/ml R-848 [0.32-26 µM] or 1 µg/ml LPS for 24 hours. *A*, The cells were subsequently stained for CD83 and CD86 cell surface expression, and the MO-DC gated population was analyzed by flow cytometry. *B*, The results are expressed as the relative cell number that stain positively within the gated population. The 30 solid lines indicate R-848 or LPS treatment, and the dotted lines indicate medium (vehicle) control. The results in *A* and *B* are representative of six independent experiments from six

different donors. C, The results are expressed as the mean fluorescence intensity (MFI) \pm SEM of six independent experiments from six different donors. $*p \leq 0.05$

Figure 2. R-848 enhances cell surface expression of co-stimulatory molecules on MO-DC. MO-DC (2×10^5) were stimulated with 2 $\mu\text{g/ml}$ R-848 for 24 hours. The cells were subsequently stained for cell surface expression of CD80, CD86, CD40, HLA-DR, CD83 and CD1a. A, The results are expressed as the relative cell number that stain positively within the MO-DC gated population and are representative of three independent experiments from three different donors. The solid lines indicate R-848 treatment, and the dotted lines indicate medium (vehicle) control. B, C, The results are expressed as the MFI \pm SEM of at least three independent experiments from three different donors. $*p \leq 0.05$

Figure 3. Maturation of monocyte-derived dendritic cells requires between 1 and 6 hours stimulation with R-848. MO-DC (2×10^5) were stimulated with 2 $\mu\text{g/ml}$ R-848 for 6 hours. A, B, The cells were subsequently stained for cell surface expression of CD80, CD86, CD40, HLA-DR, CD83 and CD1a. C, D, The cells were extensively washed, re-cultured for an additional 18 hours, and then subsequently stained for cell surface expression of CD80, CD86, CD40, HLA-DR, CD83 and CD1a. The results are expressed as MFI \pm SEM of three independent experiments from three different donors. $*p \leq 0.05$

Figure 4. T cell proliferation and T cell cytokine production are increased by R-848-treated MO-DC in a primary MLR. MO-DC (2×10^5) were stimulated with 0.1-8 $\mu\text{g/ml}$ R-848 or 1 $\mu\text{g/ml}$ LPS for 24 hours. The cells were extensively washed and cultured at graded doses with 1×10^5 CD3 enriched allogeneic T cells in triplicate. A, Proliferation was assessed by [^3H]thymidine incorporation after 96 hours. The results are expressed as mean CPM \pm SEM of three independent experiments from three different donors. Statistically significant differences ($p \leq 0.05$) were determined between R-848 [2 and 8 $\mu\text{g/ml}$] and LPS treated groups as compared to vehicle [0 $\mu\text{g/ml}$] treated group at $4-32 \times 10^3$ MO-DC. B-D, IL-2, IL-5 and IFN- γ protein were assessed from the culture supernatants as described in Materials and Methods. The results are expressed as mean $\text{pg/ml} \pm$ SEM of three independent experiments from three different donors. Statistically significant differences ($p \leq 0.05$) were determined between R-848 [2 and 8 $\mu\text{g/ml}$] and LPS treated groups as compared to vehicle [0 $\mu\text{g/ml}$] treated group at $8-32 \times 10^3$ MO-DC.

Figure 5. Autologous T cell proliferation and T cell cytokine secretion are increased by R-848-treated MO-DC in an anamnestic response to tetanus toxoid. MO-DC (2×10^5) were stimulated with 2 $\mu\text{g/ml}$ R-848 and 10 $\mu\text{g/ml}$ tetanus toxoid for 24 hours. The cells were extensively washed and cultured at graded doses with 1×10^5 CD3 enriched syngeneic T cells in triplicate for seven days. *A, B*, Proliferation was assessed by [^3H]thymidine incorporation after seven days. *C, D*, IFN- γ protein was assessed from the culture supernatants as described in Materials and Methods. The results are expressed as mean $\text{pg/ml} \pm \text{SEM}$ of three independent experiments from three different donors. The values indicated above some of the data points represent $p\text{-values} \leq 0.05$.

10

Table I. R-848 stimulates MO-DC cytokine and chemokine secretion"

Treatment [$\mu\text{g/ml}$]	IFN- α	TNF- α	IL-6	IL-8	IL-12	MCP-1	MIP-1 α
0 (vehicle)	3 \pm 2	5 \pm 2	3 \pm 2	305 \pm 77	27 \pm 11	1742 \pm 646	46 \pm 46
0.1 R-848	8 \pm 3	6 \pm 3	20 \pm 12	425 \pm 132	70 \pm 21	3603 \pm 2158	57 \pm 57
0.4 R-848	8 \pm 3	14 \pm 4	399 \pm 208	5125 \pm 2430	106 \pm 27	4864 \pm 2213	511 \pm 314
2.0 R-848	27 \pm 7*	1540 \pm 371*	6729 \pm 1888*	50092 \pm 10385*	15984 \pm 3860*	12941 \pm 5802	15412 \pm 5244*
8.0 R-848	41 \pm 12*	2208 \pm 240*	9690 \pm 1269*	66988 \pm 11863*	19640 \pm 3966*	16249 \pm 7661	25956 \pm 5782*
1.0 LPS	59 \pm 12*	2246 \pm 438*	10134 \pm 1687*	64668 \pm 12407*	15593 \pm 2755*	11006 \pm 4485	36243 \pm 8676*

* MO-DC (2×10^5) were cultured for 24 hours in cRPMI containing graded doses of R-848 or LPS at 37°C with 5% CO₂.

Culture supernatants were collected and stored at -70°C until analysis by ELISA or by bioassay. Data are given as mean \pm SEM of five independent experiments from five different donors. All values are in pg/ml, except IFN- α which is in U/ml.

*, $p \leq 0.05$, as compared to cytokine levels in vehicle control.

Table II. *TNF- α and IL-12 production from MO-DC requires between 1 and 6 hours stimulation with R-848^a*

Treatment time (hr) ^b	Treatment ^c	TNF- α	IL-12
1	vehicle	1 \pm 1	73 + 37
	R-848	32 \pm 10*	65 + 18
	LPS	51 + 8*	44 + 19
6	vehicle	3 + 3	92 + 99
	R-848	1053 + 707*	4446 + 2438*
	LPS	2679 + 557*	6160 + 1109*
24	vehicle	3 + 4	107 + 32
	R-848	335 + 201*	13153 + 5484*
	LPS	1675 + 665*	21167 + 1050*

^a MO-DC (2×10^5) were cultured for 24 hours in cRPMI containing graded doses of R-848 or LPS at 37°C with 5% CO₂. Culture supernatants were collected and stored at -70°C until analysis by ELISA. Data are given as mean pg/ml \pm SEM of three independent experiments from three different donors.

^b Treatment time (hr) is the length of time MO-DC were in culture with R-848 or LPS.

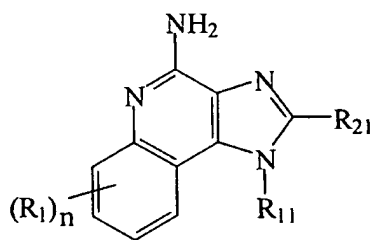
^c MO-DC were treated for the indicated times with 2 μ g/ml R-848, 1 μ g/ml LPS or vehicle (PBS).

*, $p \leq 0.05$, as compared to the cytokine levels in the vehicle control.

The present invention has been described with reference to several embodiments thereof. The foregoing detailed description and examples have been provided for clarity of understanding only, and no unnecessary limitations are to be understood therefrom. It will be apparent to those skilled in the art that many changes can be made to the described
5 embodiments without departing from the spirit and scope of the invention. Thus, the scope of the invention should not be limited to the exact details of the methods, compositions and structures described herein, but rather by the language of the claims that follow.

WHAT IS CLAIMED IS:

1. A method of *in vitro* maturation of immature dendritic cells comprising stimulating said immature dendritic cells with an imidazoquinoline type immune response modifying compound.
2. The method of claim 1 wherein the immature dendritic cells are monocyte-derived dendritic cells.
3. The method of claim 1 wherein the immature dendritic cells are obtained by incubating human peripheral blood mononuclear cells with GM-CSF and IL-4.
4. The method of claim 1 wherein the imidazoquinoline type immune response modifying compound comprises a 1H-imidazo[4,5-c] quinoline-4-amine.
5. The method of claim 1 wherein the imidazoquinoline type immune response modifying compound is a compound of the formula:



I

wherein

- R_{11} is selected from the group consisting of alkyl of one to ten carbon atoms, hydroxyalkyl of one to six carbon atoms, acyloxyalkyl wherein the acyloxy moiety is alkanoyloxy of two to four carbon atoms or benzoyloxy, and the alkyl moiety contains one to six carbon atoms, benzyl, (phenyl)ethyl and phenyl, said benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms and halogen, with the proviso that if said benzene ring

is substituted by two of said moieties, then said moieties together contain no more than six carbon atoms;

5 R₂₁ is selected from the group consisting of hydrogen, alkyl of one to eight carbon atoms, benzyl, (phenyl)ethyl and phenyl, the benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms and halogen, with the proviso that when the benzene ring is substituted by two of said moieties, then the moieties together contain no more than six carbon atoms; and

10 each R₁ is independently selected from the group consisting of alkoxy of one to four carbon atoms, halogen, and alkyl of one to four carbon atoms, and n is an integer from 0 to 2, with the proviso that if n is 2, then said R₁ groups together contain no more than six carbon atoms; or a pharmaceutically acceptable salt or solvate thereof.

6. The method of claim 1 wherein the imidazoquinoline type immune response
15 modifying compound is 4-amino-2-ethoxymethyl- α,α -dimethyl-1H-imidazo[4,5-c]quinolin-1-ethanol.

7. The method of claim 1 wherein the resulting mature dendritic cells induce at least a two- fold increase in the proliferation of naïve allogeneic T-cells and/or display at least a three- fold increase in the production of dendritic cell cytokines.

20 8. The method of claim 1 wherein the immature dendritic cells are stimulated for about 16 to about 24 hours.

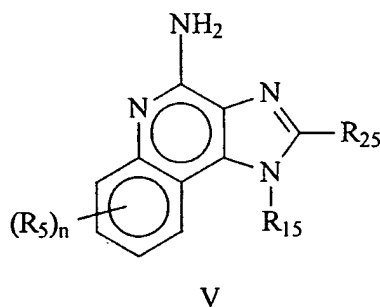
9. A population of mature dendritic cells produced by the method of claim 1.

10. A method of enhancing the antigen presenting ability of dendritic cells comprising stimulating said dendritic cells with an imidazoquinoline type immune response
25 modifying compound.

11. A method of preparing a cellular adjuvant for the treatment of a disease comprising:

- 30 (a) maturing dendritic cells *in vitro* by treating the dendritic cells with an imidazoquinoline type immune response modifying compound and
 (b) exposing the mature dendritic cells to an antigen associated with said disease.

12. The method of claim 11 wherein the disease is a neoplastic disease and the antigen is derived from neoplastic cells.
13. The method of claim 11 wherein the disease is caused by an infectious agent and the antigen is derived from the infectious agent.
- 5 14. The method of claim 11 wherein the antigen is recombinantly derived.
15. A method of treating a disease comprising administering a therapeutically effective dose of the cellular adjuvant of claim 11 to a mammal in need of such treatment.
16. A method of treating a disease comprising administering a therapeutically effective dose of dendritic cells that have been matured by stimulation with an
- 10 imidazoquinoline type IRM to mammal in need of such treatment.
17. The method of claim 16 wherein the disease is a neoplastic disease.
18. The method of claim 16 wherein the disease is a Th2 mediated disease.
19. A cellular adjuvant prepared by the method of claim 11.
20. The method of claim 1 wherein the imidazoquinoline type immune response
- 15 modifying compound is a compound of the formula:



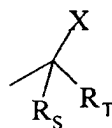
20 wherein

R_{15} is selected from the group consisting of: hydrogen; straight chain or branched chain alkyl containing one to ten carbon atoms and substituted straight chain or branched chain alkyl containing one to ten carbon atoms, wherein the substituent is selected from the group consisting of cycloalkyl containing three to six carbon atoms and cycloalkyl

25 containing three to six carbon atoms substituted by straight chain or branched chain alkyl containing one to four carbon atoms; straight chain or branched chain alkenyl containing

two to ten carbon atoms and substituted straight chain or branched chain alkenyl containing two to ten carbon atoms, wherein the substituent is selected from the group consisting of cycloalkyl containing three to six carbon atoms and cycloalkyl containing three to six carbon atoms substituted by straight chain or branched chain alkyl containing one to four carbon atoms; hydroxyalkyl of one to six carbon atoms; alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to six carbon atoms; acyloxyalkyl wherein the acyloxy moiety is alkanoyloxy of two to four carbon atoms or benzoyloxy, and the alkyl moiety contains one to six carbon atoms; benzyl; (phenyl)ethyl; and phenyl; said benzyl, (phenyl)ethyl or phenyl substituent being optionally substituted on the benzene ring by one or two moieties independently selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms, and halogen, with the proviso that when said benzene ring is substituted by two of said moieties, then the moieties together contain no more than six carbon atoms;

R₂₅ is



wherein

R_S and R_T are independently selected from the group consisting of hydrogen, alkyl of one to four carbon atoms, phenyl, and substituted phenyl wherein the substituent is selected from the group consisting of alkyl of one to four carbon atoms, alkoxy of one to four carbon atoms, and halogen;

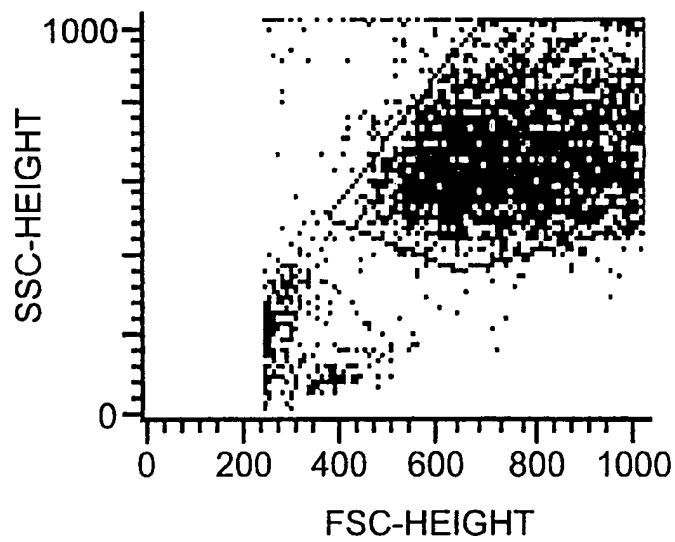
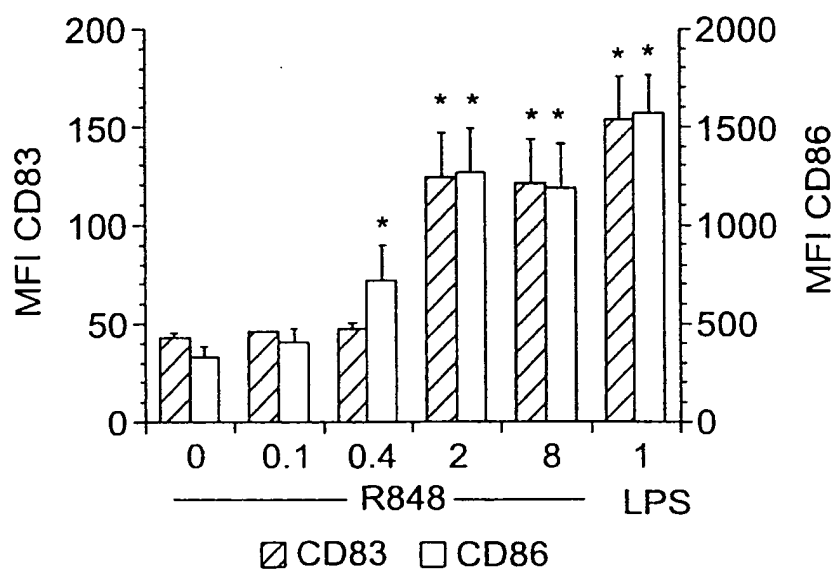
X is selected from the group consisting of alkoxy containing one to four carbon atoms, alkoxyalkyl wherein the alkoxy moiety contains one to four carbon atoms and the alkyl moiety contains one to four carbon atoms, hydroxyalkyl of one to four carbon atoms, haloalkyl of one to four carbon atoms, alkylamido wherein the alkyl group contains one to four carbon atoms, amino, substituted amino wherein the substituent is alkyl or

hydroxyalkyl of one to four carbon atoms, azido, chloro, hydroxy, 1-morpholino, 1-pyrrolidino, alkylthio of one to four carbon atoms; and

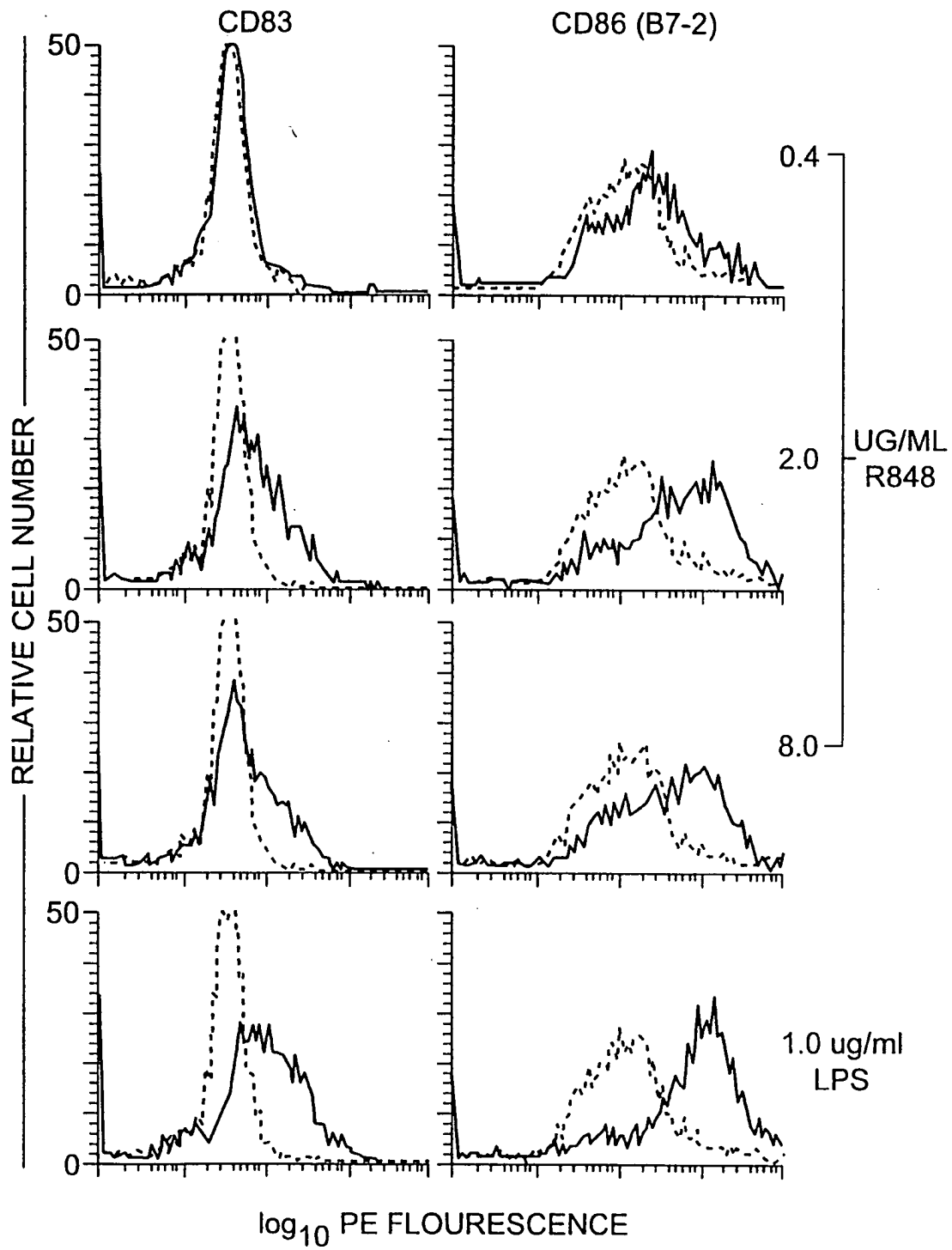
5 R_5 is selected from the group consisting of hydrogen, straight chain or branched chain alkoxy containing one to four carbon atoms, halogen, and straight chain or branched chain alkyl containing one to four carbon atoms, and n is an integer from 0 to 2, with the proviso that if n is 2, then said R_5 groups together contain no more than six carbon atoms, or a pharmaceutically acceptable salt or solvate thereof.

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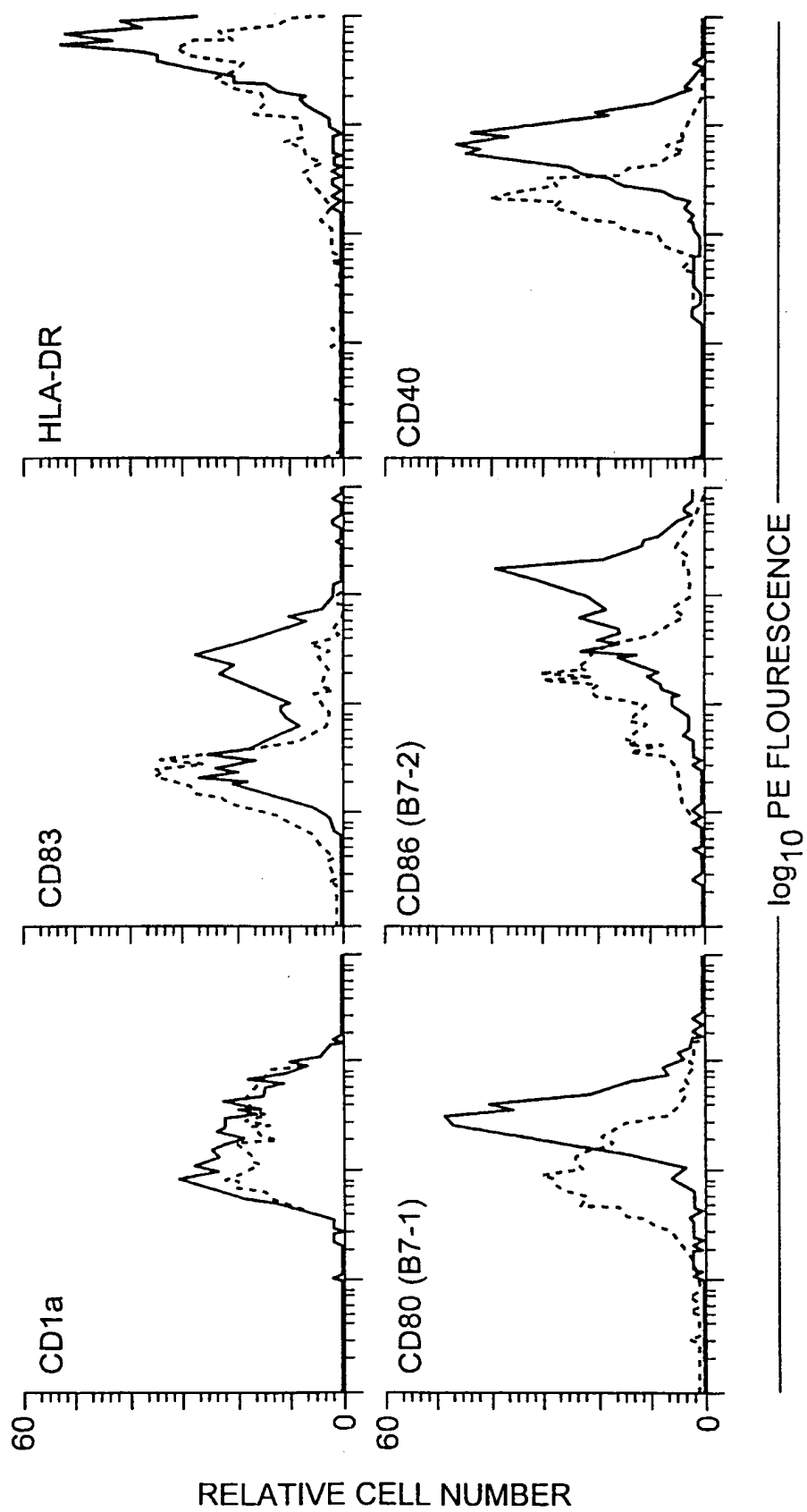
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*Fig. 1A**Fig. 1C*

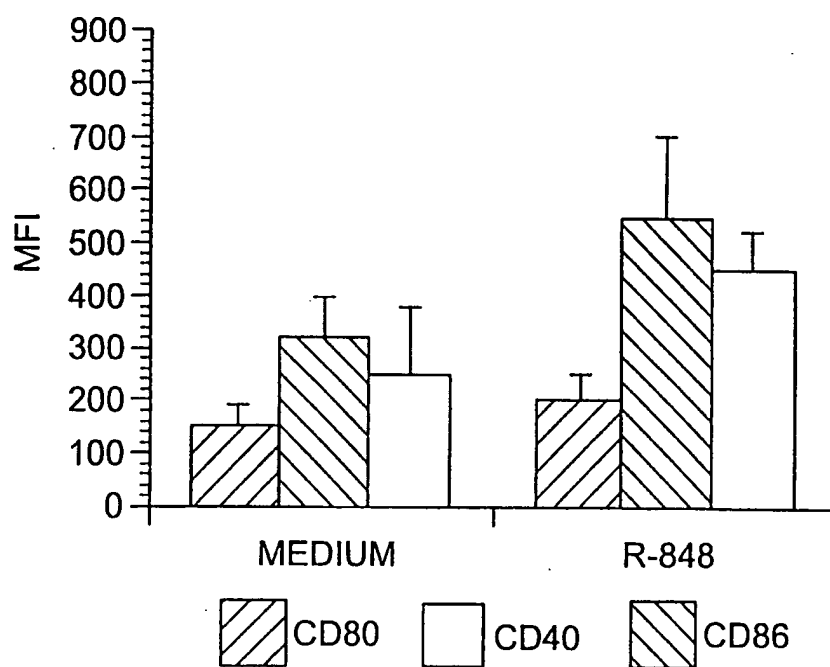
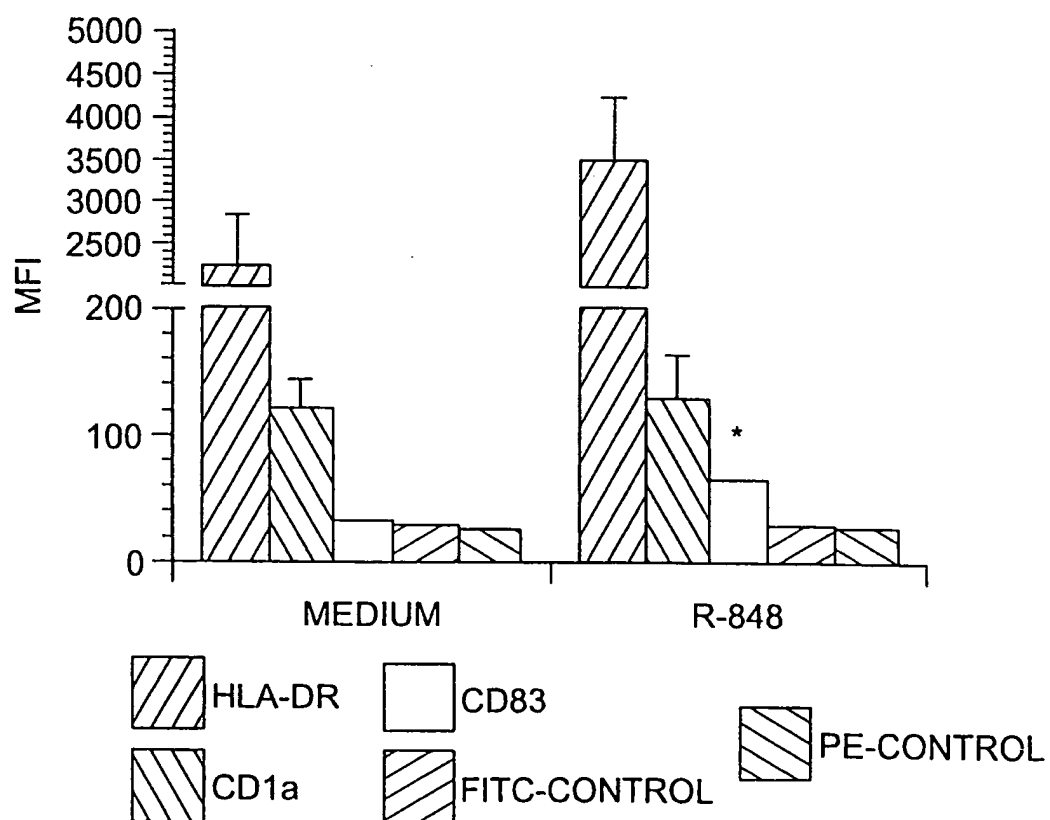
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**Fig. 1B**

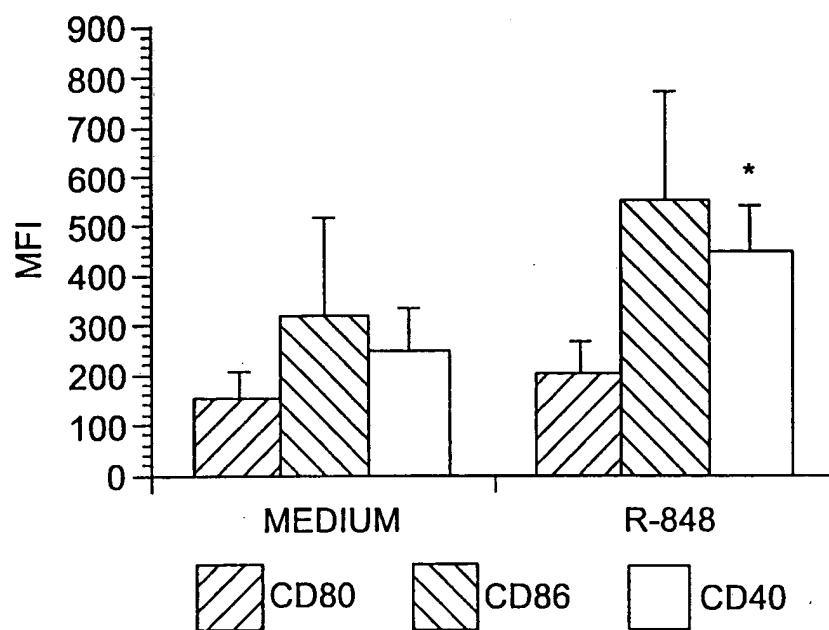
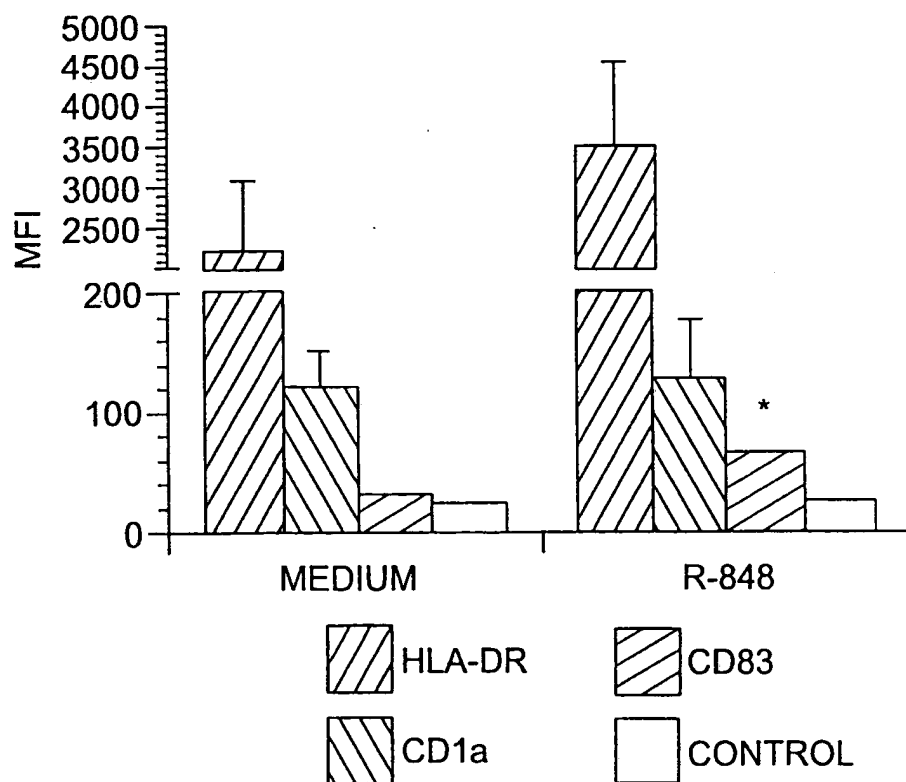
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*Fig. 2A*

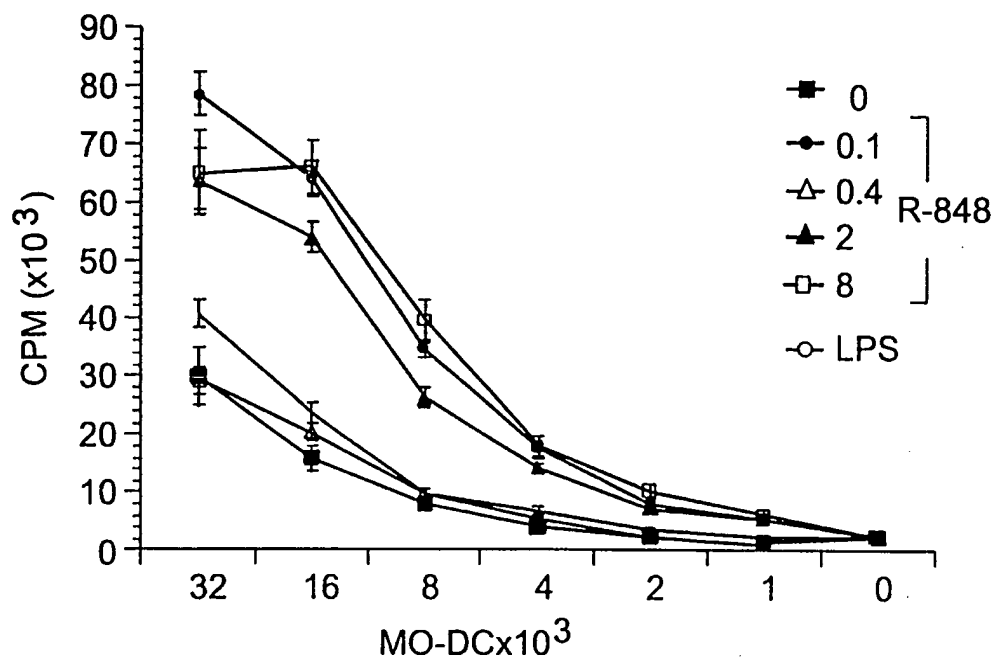
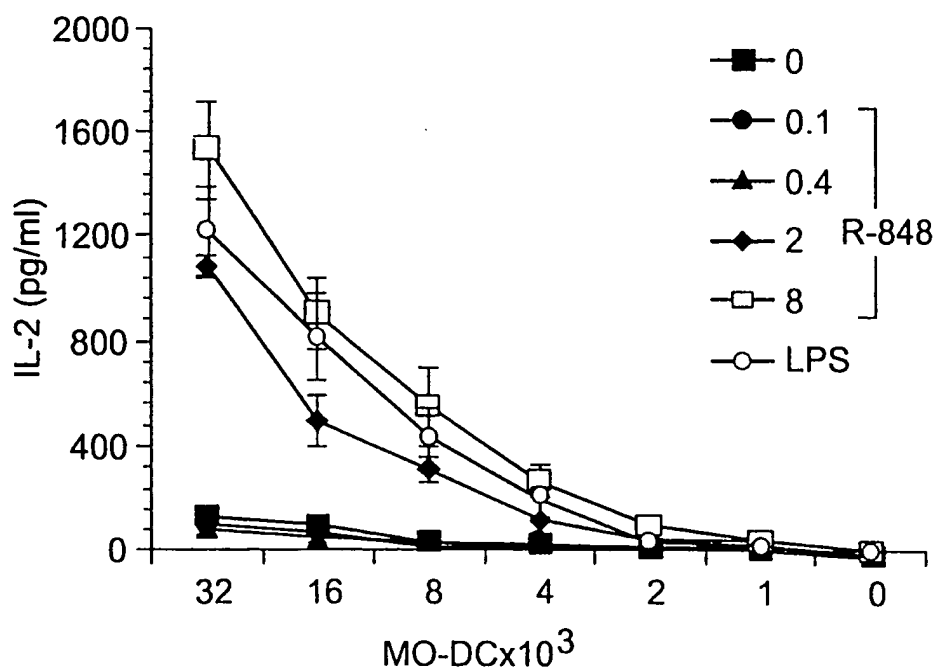
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*Fig. 3A**Fig. 3B*

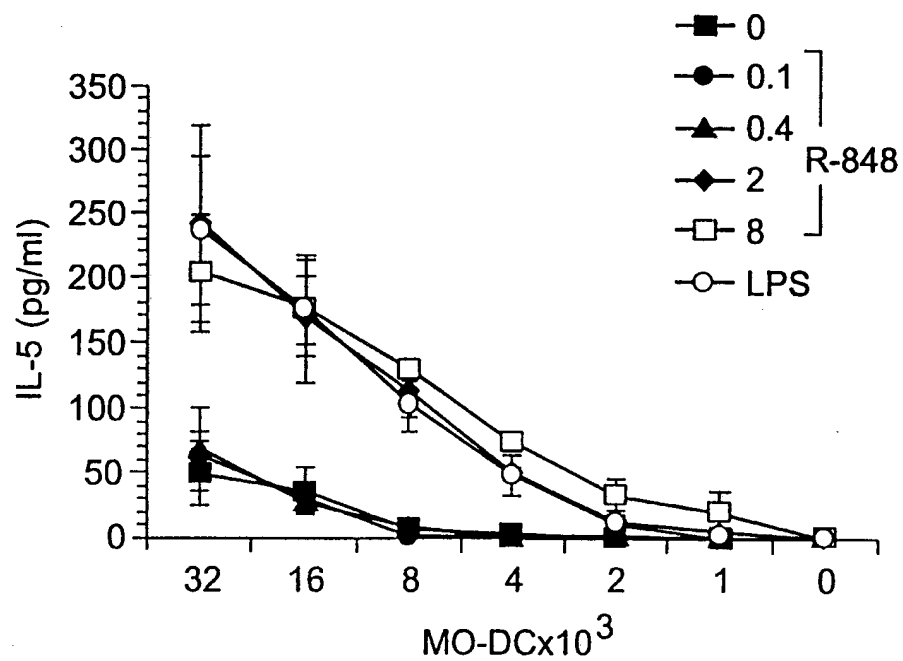
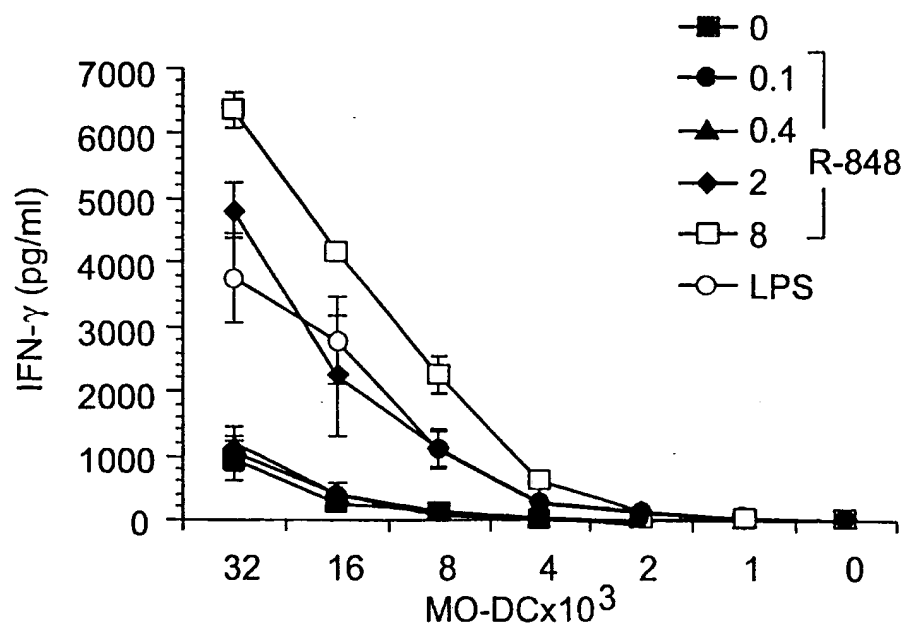
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*Fig. 3C**Fig. 3D*

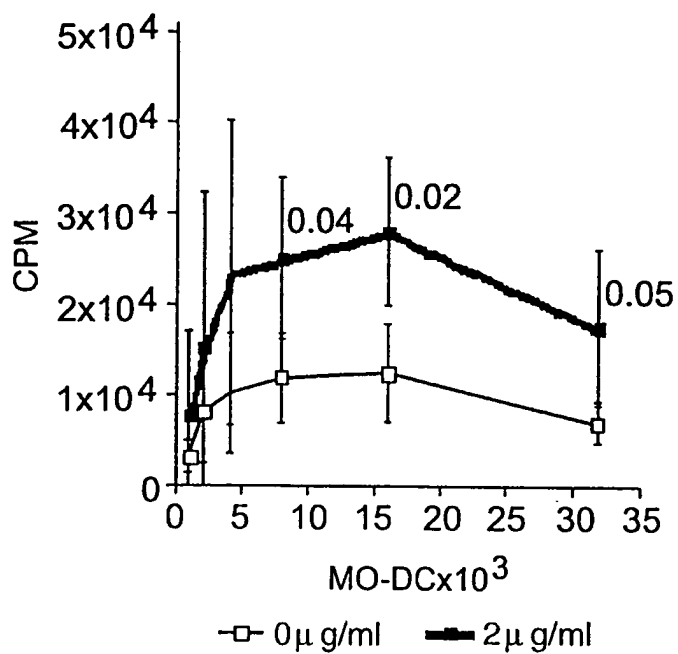
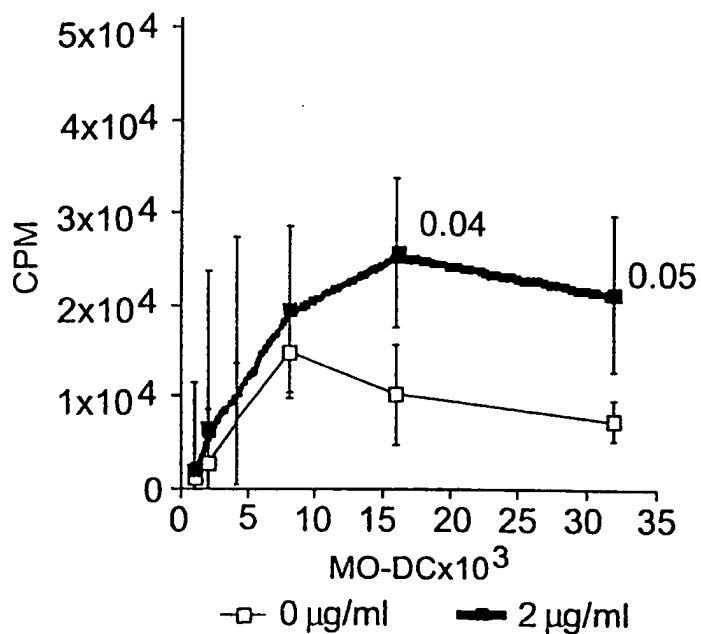
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*Fig. 4A**Fig. 4B*

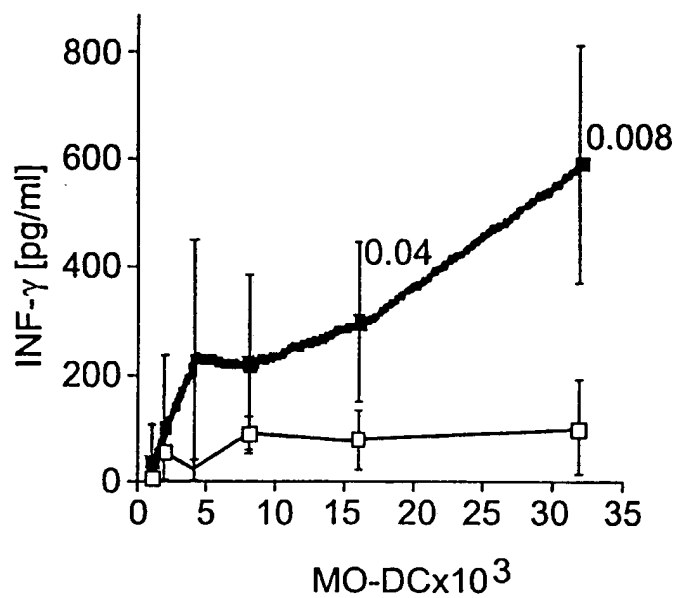
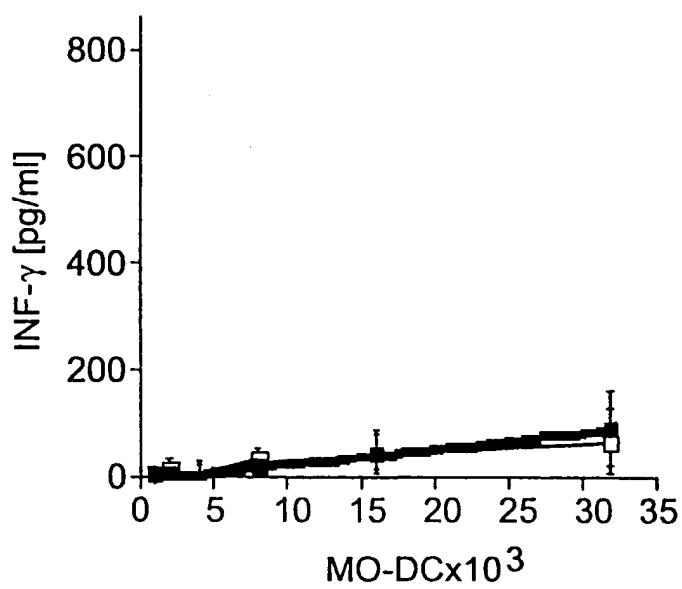
7/9

*Fig. 4C**Fig. 4D*

8/9

*Fig. 5A**Fig. 5B*

9/9

*Fig. 5C**Fig. 5D*